THE IMPACT OF CHEMOTHERAPY ON THE HOST MICROBIOTA IN THE CONTEXT OF ORAL AND GASTROINTESTINAL MUCOSITIS

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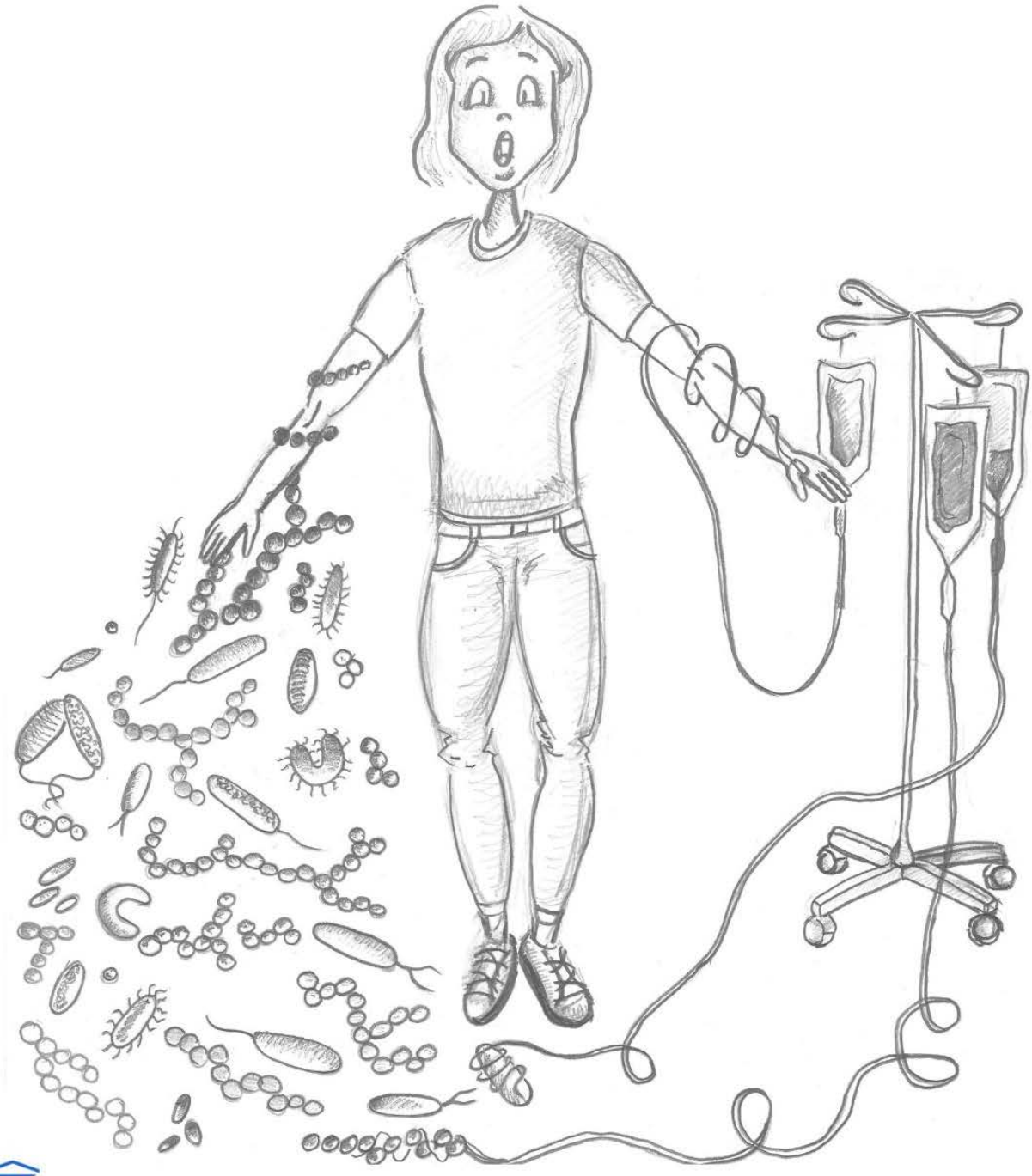

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The impact of chemotherapy on the host microbiota in the context of oral and gastrointestinal mucositis

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Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences at Ghent University

Titel van het doctoraat in het Nederlands

De impact van chemotherapie op de gastheer microbiota in de context van orale en gastrointestinale mucositis.

Cover illustration

Elien Audenaert

Please refer to this work as:

Vanlancker, E. (2017). The impact of chemotherapy on the host microbiota in the context of oral and gastrointestinal mucositis. PhD thesis, Ghent University, Belgium

ISBN 978-94-6357-036-7

This work was supported by the Special Research Fund (Bijzonder OnderzoeksFonds (BOF)) of Ghent University.

Notation Index

5-FU	5-Fluorourcil
AML	Acute Myeloid Leukemia
AV	Average
CFU	Colony-forming units
DAMP	Damage-associated molecular pattern
DGGE	Denaturing gradient gel electrophoresis
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPD	Dihydropyrimidine dehydrogenase
EDTA	Ethylenediaminetetraacetic acid
HSCT	Hematopoietic Stem Cell Transplantation
IL	Interleukin
LLLT	Low Level Laser Therapy
(M-)SHIME	Mucosal simulator of the human intestinal microbial ecosystem
MAMP	Microbe-associated molecular pattern
MAPK	Mitogen-activated protein kinase
MASCC/ISOO	Multinational Association of Supportive Care in Cancer / International Society of Oral Oncology
MMP	Matrix metalloproteinase
MTT	3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyl tetrazolium bromide
MTX	Methotrexate
NF-κB	Nuclear Factor-kappa B
NLR	Nucleotide-binding oligomerization domain (NOD)-like receptors
NMDS	Non-metric multidimensional scaling
OTU	Operational taxonomic unit
PBS	Phosphate buffered solution
PCR	Polymerase chain reaction
PRR	Pattern recognition receptor

(r)RNA	(ribosomal) Ribonucleic acid
ROS	Reactive oxygen species
SCFA	Short-chain fatty acids
SD	Standard deviation
SN-38	7-Ethyl-10-hydroxy-camptothecin
SRB	Sulforhodamine B
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TS	Thymidylate synthase
WHO	World Health Organization

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CHAPTER 1

Introduction

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1. Mucositis

Mucositis is a common side effect of cancer treatment with a major impact on the quality of life of patients and occurring during all types of cancer treatment: chemotherapy, radiotherapy and targeted therapy (Sonis 2009, Al-Dasooqi et al. 2013, Lalla et al. 2014, Villa and Sonis 2015). It is an inflammation and ulceration of the mucosal surfaces and can occur in all types of mucosa. Oral and gastrointestinal mucositis (including esophagus, stomach, small and large intestine) are the most prominent, but also the respiratory and genitourinary tract can be affected by cytotoxic drugs (Peterson et al. 2012, Al-Dasooqi et al. 2013). The general term 'alimentary mucositis' is often used to describe mucositis in the entire alimentary tract (mouth to anus) (Keefe et al. 2007).

1.1 Oral mucositis

Oral mucositis is defined as injuries in the oral cavity following cancer treatment and is one of the best-studied side effects of cancer treatment (Al-Dasooqi et al. 2013). The major symptoms are oral pain, erythema, edema and ulcerations causing a higher infection risk which can lead to bacteremia, fungemia and sepsis (Sonis 2004, Potting et al. 2006, Peterson et al. 2012). Oral mucositis also majorly impact the quality of life of the patients *i.e.* problems with speaking and intake of food and medications which may lead to a dose reduction of the cancer treatment and relapse of the cancer (Sonis 2004, Barasch and Epstein 2011). In order to estimate the severity of oral mucositis, different scales are used. The widely used World Health Organization (WHO) scale combines functional (ability to eat) and objective (erythema, ulceration) measurements of oral mucositis (Villa and Sonis 2015) (Table 1.1).

Table 1.1 - World Health Organization (WHO) grading scale for oral mucositis (WHO 1979)

Grade	Clinical measurements
0	No symptoms
1	Pain, erythema
2	Erythema, ulcers, able to eat solids
3	Confluent ulcers, unable to eat solids, liquid diet possible
4	Severe ulceration, bleeding, oral alimentation not possible

The incidence of oral mucositis is high but varies depending on the type of treatment and is often underreported. The reported frequency of oral mucositis is around 20 to 40 % in conventional chemotherapy for solid tumors, around 80 % for high-dose chemotherapy prior to hematopoietic stem cell transplantation (HSCT), but reaching 100 % for radiotherapy for head and neck cancer (Sonis 2007, Lalla et al. 2008, Villa and Sonis 2015). Next to the type of cancer treatment also the type of cancer drugs, dosage, delivery schedule and chemotherapy cycle are important therapy-related risk factors (Villa and Sonis 2015).

Despite the large number of people suffering from mucositis and the major impact on their quality of life, only limited treatment options are available so far. Only palifermin (keratinocyte growth factor-1 (KGF-1)) is approved by the US Food and Drug Administration and the European Medicines Agency for treatment of oral mucositis in patients receiving high-dose chemotherapy and total body irradiation, followed by HSCT for hematological malignancies (Sonis 2007). The Multinational Association for Supportive Care in Cancer and International Society of Oral Oncology (MASCC/ISOO) formulated guidelines for the prevention and treatment of oral mucositis, including the use of palifermin to prevent oral mucositis induced by conditioning regimens for HSCT. Recommendations to prevent oral mucositis in specific groups of patients include the use of cryotherapy, low-level laser therapy (LLLT) and benzydamine mouthwash (Lalla et al. 2014). The use of good oral care protocols (typically including brushing, flossing, rinsing and moisturizing) may also reduce the risk, length and severity of oral mucositis and lower the risk of infection by reducing the bacterial load (McGuire et al. 2013, Villa and Sonis 2015). An interdisciplinary and educational approach is important to assure the application of these good oral practices (Keefe et al. 2007). To treat the pain, the use of different mouthwashes based on morphine and doxepin is suggested for specific groups of patients (Lalla et al. 2014).

1.2 Gastrointestinal mucositis

Gastrointestinal mucositis includes inflammation and/or ulceration of the gastrointestinal mucosa along the entire alimentary tract (except the oral cavity), but is most prominent in the small intestine (Sonis et al. 2004a). Symptoms can include abdominal pain, diarrhea, constipation, bleeding, abdominal bloating, malnutrition, dehydration, electrolyte imbalance and infections (Gibson and Keefe 2006, Touchefeu et al. 2014). All these symptoms impact patient's quality of life and can lead to a reduction or delay in cancer treatment, increased costs due to longer hospitalization and symptom management and even lower survival rates (Elting et al. 2003, Gibson et al. 2013, Lalla et al. 2014). In contrast to oral mucositis, only limited scales are available to assess the severity of gastrointestinal mucositis. Due to the inaccessibility of the small and large intestine, these scales typically measure indirect outcomes of mucosal injury, including diarrhea (Peterson et al. 2011). One commonly used

scale is the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events (CTCAE) scale which can be used for diarrhea and abdominal pain (Peterson et al. 2015) (Table 1.2).

Table 1.2 - National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events (CTCAE) scale for diarrhea (defined as a disorder characterized by frequent and watery bowel movements) and abdominal pain (National Cancer Institute 2009).

Grade	Clinical measurements
Diarrhea	
0	No symptoms
1	Increase of < 4 stools per day over baseline; mild increase in ostomy output compared with baseline
2	Increase of 4–6 stools per day over baseline; moderate increase in ostomy output compared with baseline
3	Increase of ≥ 7 stools per day over baseline; incontinence; hospitalization indicated; severe increase in ostomy output compared with baseline; limiting self-care activities of daily living (ADL)
4	Life-threatening consequences; urgent intervention indicated
5	Death
Abdominal pain	
0	No symptoms
1	Mild pain
2	Moderate pain; limiting instrumental activities of daily living (ADL)
3	Severe pain; limiting self care activities of daily living (ADL)

Gastrointestinal mucositis is associated with many commonly used chemotherapy regimens (Sonis et al. 2004a), but the incidence is highly dependent on the type of treatment regimen and chemotherapeutic agent. Cancer treatments containing fluoropyrimidines (such as 5-fluorouracil (5-FU)) and/or irinotecan have an incidence of chemotherapy-induced diarrhea as high as 50 - 80 % (Benson et al. 2004, Gibson and Stringer 2009). Similar to oral mucositis, only limited options are available to prevent or treat gastrointestinal mucositis. As a first line, loperamide is used to treat chemotherapy-induced diarrhea (Benson et al. 2004). Moreover, the MASCC/ISOO guidelines recommend and/or suggest the use of amifostine, sulfasalazine and probiotics containing *Lactobacillus* for prevention and octreotide, sucralflate and hyperbaric oxygen for treatment in specific groups of patients (Lalla et al. 2014). Glutamine and antibiotics have not been shown to be effective (Lalla et al. 2014, Touchefeu et al. 2014).

1.3 Pathobiology of mucositis

Historically, mucositis was thought to be solely an epithelial injury. As cancer therapy targets rapidly dividing cancer cells, also rapidly dividing normal cells will get damaged, such as the basal epithelial cells in the gastrointestinal and oral mucosa (Sonis 2004, Sonis 2007). In 2004, however, Sonis described mucositis as a more complex process, existing of 5 phases and comprising not only the epithelium but also other compartments of the mucosa (Sonis 2004, Sonis 2007, Sonis 2009). This model is applicable to the entire alimentary tract, so to both oral and gastrointestinal mucositis (Sonis 2004).

In the **initiation phase** chemotherapy will cause DNA damage and strand breaks, which may lead to cell death in the epithelium and the submucosa, and the generation of reactive oxygen species (ROS). Chemotherapy, radiation and ROS will further activate different pathways during the **primary response phase** (Sonis 2004, Sonis 2009). One of the most significant and best-studied activated transcription factors is nuclear factor-kappa B (NF-κB). It regulates the expression of more than 200 genes and will for example stimulate the production of pro-inflammatory cytokines such as Tumor Necrosis Factor (TNF), Interleukin-1β (IL-1β) and IL-6. Moreover, NF-κB impacts genes of the BCL2 family, controlling apoptosis (Sonis 2002, Sonis 2007, Logan et al. 2008b). Cell membranes can also be disrupted by activation of sphingomyelinase or ceramide synthase, leading to apoptosis (Hwang et al. 2005). Moreover, stimulation of secretion of matrix metalloproteinases (MMPs) will cause break down of the extracellular matrix in the submucosa (Sonis 2007). More recently the production of Damage-Associated Molecular Patterns (DAMPs) by damaged cells due to chemotherapy and radiotherapy was also proposed to be crucial in the early stages of mucositis. These DAMPS will bind to Pattern Recognition Receptors (PRR) which in turn will promote the activation of the NF-κB pathway (Sonis 2010).

In the **signal amplification phase**, the proteins produced during the primary damage, particularly pro-inflammatory cytokines, will stimulate additional damage by positive feedback loops. For example, TNF stimulates NF-κB and activates mitogen-activated protein kinase (MAPK) signaling, which can ultimately result in cell death (Sonis 2002, Sonis 2004). Again, TNF can repeatedly activate sphingomyelinases (Andrieu-Abadie et al. 2001) and MMPs leading to accumulated damage (Sasaki et al. 2000). Although many biological processes are activated during the early stage of oral mucositis, the epithelium is still intact and only few symptoms are visual, generally only erythema (Sonis 2004). In contrast, the **ulceration phase** is the most significant phase both at a symptomatic as at a clinical level. The loss of mucosal integrity leads to the formation of ulcerative lesions covered with a pseudomembrane. Bacteria will colonize these lesions and penetrate the submucosa which can lead to bacteremia or sepsis, mostly in neutropenic patients. In addition, bacterial cell-wall products penetrated into

the submucosa, will stimulate production of pro-inflammatory cytokines by macrophages (Sonis 2004, Sonis 2007). Last, the **healing phase** is probably still the least understood. Healing occurs spontaneously and is regulated by signals from the extracellular matrix leading to migration, proliferation and differentiation of epithelial cells (Sonis 2004, Sonis 2007). Also cyclo-oxygenase-2 (COX-2) may play a role as it stimulates angiogenesis (Sonis et al. 2004b, Logan et al. 2007). However, the structure of the submucosa will never return to the initial state (Denham and Hauer-Jensen 2002, Sonis 2007).

2. Chemotherapy

Chemotherapy mostly leads to an acute form of mucositis (in comparison with radiotherapy which causes chronic mucositis), developing within 3-4 days after initiation of chemotherapy and peaking within 2 weeks (Raber-Durlacher et al. 2010). High risk for development of oral mucositis exists for 5-FU, methotrexate, doxorubicin, cytarabine, cisplatin, cyclophosphamide and etoposide (Naidu et al. 2004, Napenas et al. 2007, Qutob et al. 2013, Villa and Sonis 2015). Gastrointestinal mucositis and its associated diarrhea are mostly associated with drugs like fluoropyrimidines (5-FU or capecitabine) and irinotecan but it also occurs in treatment regimens based on other chemotherapeutic agents (Wadler et al. 1998, Benson et al. 2004). Although the focus in this dissertation is concentrated on the effects of 5-FU and irinotecan, also other chemotherapeutic agents causing mucositis are shortly reviewed here.

2.1 5-Fluorouracil

5-FU is one of the oldest chemotherapeutic agents and is used to combat a wide range of cancer types, including colorectal and breast cancer. This pyrimidine derivate is an analogue of uracil with a fluorine atom on the C-5 position (Longley et al. 2003). After entering the cell via facilitated transport, three active metabolites are produced: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP) which all lead to DNA and RNA damage via misincorporation into DNA and RNA and inhibition of thymidylate synthase (TS) (Grem 2000, Longley et al. 2003). In the liver, however, 80 % of the 5-FU dose is catabolized into the inactive metabolite dihydrofluorouracil (DHFU) by dihydropyrimidine dehydrogenase (DPD) (Diasio and Harris 1989, Longley et al. 2003), contributing to the short half-life time of 5-FU (6-22 min) (Bocci et al. 2000). To increase the efficacy, 5-FU is mostly combined with leucoverin (folinic acid), which increases intracellular levels of reduced folate. The active metabolite fluorodeoxyuridine monophosphate (FdUMP) needs this reduced folate as a cofactor to inhibit TS (Johnston and Kaye 2001, Longley et al. 2003). Another approach is to the use oral 5-FU prodrugs

capecitabine, to avoid DPD deactivation in the liver. These prodrugs will be absorbed in the gastrointestinal tract and will be preferentially converted to 5-FU in the tumor, resulting in an improved efficacy (Johnston and Kaye 2001, Longley et al. 2003).

Next to leukopenia and thrombocytopenia, 5-FU causes both oral and gastrointestinal mucositis (Stringer et al. 2009b). The incidence is about 40 % for oral mucositis and 10 – 15 % for grade 3 or 4 oral mucositis (Rubenstein et al. 2004, Sonis et al. 2004a). However, in specific regimens the incidence of grade 3 or 4 oral mucositis can reach 66 % for 5-FU in combination with other chemotherapeutics (Sonis et al. 2004a). In colorectal cancer the incidence for grade 3 or 4 diarrhea is 10 % for treatment with FOLFOX (Folinic acid, 5-FU and Oxaliplatin) or FOLFIRI (Folinic acid, 5-FU and irinotecan) (Jones et al. 2006). However, other studies show incidences reaching 38 % for grade 3 and 4 diarrhea for regimens including 5-FU (Sonis et al. 2004a). It has been shown that several factors can modulate the mucositis risk. First, DPD deficiency will increase 5-FU plasma concentration, leading to higher toxicity rates (Diasio et al. 1988, Harris et al. 1991), whereas tumor-selective activation of prodrugs as capecitabine will lead to lower toxicity profiles (Johnston and Kaye 2001, Longley et al. 2003). Although continuous infusion of 5-FU is better tolerated than bolus in general, both oral and gastrointestinal mucositis occur with high incidences in both regimens (Johnston and Kaye 2001). After an intravenous bolus injection with 5-FU, plasma concentrations in cancer patients can reach some hundreds µM, but the plasma levels drop to 15-30 µM after 30 minutes and to 0 µM after 2 h (Casale et al. 2004, Kosovec et al. 2008), due to the short half life time of 5-FU (Bocci et al. 2000). In case of continuous infusion with 5-FU, the plasma concentrations are much lower, ranging from 3 to 10 µM and kept for a longer time period (24 h) (Joulia et al. 1999, Takimoto et al. 1999). Levels in saliva during this continuous infusion range between 0.08 and 0.8 µM (Joulia et al. 1999).

2.2 Irinotecan

Irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin, CPT-11) is primarily used for treatment of colorectal cancer in combination with 5-FU or oxaliplatin (Jones et al. 2006, Smith et al. 2006). DNA topoisomerase is the target of this campothecin derivative (Hsiang et al. 1985). Irinotecan will bind to the DNA-topoisomerase I complex, preventing religation of the single strand breaks made by this enzyme. This will lead to irreversible double strand breaks and cell death (Smith et al. 2006). SN-38 (7-ethyl-10-hydroxycamptothecin) has been shown to be more than 100 times more active, compared to irinotecan (Kawato et al. 1991). This active metabolite is produced in the liver but can be de-activated by uridine 5'-diphospho-glucuronosyltransferase 1A1 to SN-38G (Kehrer et al. 2001). However, this SN-38G can be re-activated again to SN-38 by β-glucoronidase produced by microbiota in the colon and cause diarrhea (Takasuna et al. 1996). On average 1.2 % of

the dose is excreted as SN-38 in the feces within 24 h (Sparreboom et al. 1998, Slatter et al. 2000) and based on these data, an average colon concentration of 1-2 µM SN-38 could be estimated. Diarrhea is one of most important side effects of treatment with irinotecan, with incidences of grade 3 or 4 diarrhea of 10 % for FOLFIRI (Folinic acid, 5-FU and irinotecan) and 24 % for IROX (irinotecan and oxaliplatin) regimens for colorectal cancer (Jones et al. 2006). Other studies with irinotecan showed incidences ranging between 6 and 38 % for grade 3 or 4 diarrhea depending on the regimen (Sonis et al. 2004a).

2.3 Other chemotherapeutic agents

Methotrexate (MTX, 4-amino-4-deoxy-N10-methyl pteroyl-glutamic acid), is an analog of folic acid used in the treatment of leukemia, non-Hodgkin lymphoma's and some solid tumors (Deeming et al. 2005). MTX will interfere in the folate pathway, ultimately leading to blockage in synthesis of DNA, RNA, thymidylates and proteins (Rajagopalan et al. 2002, Vanhoecke et al. 2015a). A risk for grade 3 and 4 oral mucositis of 16-30 % is reported in pediatric patients (Sonis et al. 2004a). Salivary concentrations for MTX range between 0.01 and 0.1 µM during the first 48 h after administration (Steele et al. 1979, Schroder et al. 1987).

Doxorubicin and **daunorubicin** are anthracyclines and natural products extracted from *Streptomyces peucetiusor* or *Streptomyces galilaeus* (Nussbaumer et al. 2011). Different mechanisms are proposed for both chemotherapeutic agents of which the most important one is the interference with the uncoiling of DNA by inhibition of the topoisomerase II (Gewirtz 1999, Tacar et al. 2013, Yang et al. 2014). Doxorubicin is widely used for treatment of solid tumors and acute leukemia (Yang et al. 2014). Daunorubicin is an important agent in the treatment of acute lymphoblastic and myeloid leukemia (Nussbaumer et al. 2011). Regimens including doxorubicin have varying incidence of grade 3 and 4 oral mucositis (0-27 %) and grade 3 and 4 diarrhea (0.3-9 %) (Sonis et al. 2004a, Jones et al. 2006, Keefe et al. 2007). Maximal salivary concentrations of doxorubicin range between 0.01 and 0.15 µM (Bressolle et al. 1992).

Etoposide was the first agent targeting topoisomerase II, approved by the FDA in 1983 (Hande 1998). It is a semisynthetic glucoside of epipodophyllotoxin which will inhibit the enzyme responsible for the unwinding and cutting of the double-stranded DNA, hereby inducing cell death (Hande 1998, Nussbaumer et al. 2011). Etoposide is frequently used for treatment of small-cell bronchial carcinoma, testicular cancer and some lymphomas (Nussbaumer et al. 2011). Incidences of grade 3 and 4 oral mucositis vary from 4 to 31 % for regimens including etoposide (Sonis et al. 2004a, Jones et al. 2006, Keefe et al. 2007). Salivary concentrations of etoposide range between 0.1 and 1.5 µM during the first 12 h after administration (Holthuis et al. 1986).

Cytarabine, also known as cytosine arabinoside, is a pyrimidine analogue. Its active form cytarabine-5′-triphosphate can be misincorporated into DNA and is also a weak inhibitor

of DNA polymerase, finally leading to apoptosis (Shelton et al. 2016). It is mostly used in combination with a topoisomerase II inhibitor in treatment of leukemia and non-Hodgkin lymphoma (Bolwell et al. 1988, Shelton et al. 2016). Some regimens for hematological malignancies including cytarabine can cause oral mucositis (all grades) in 50-75 % of the cases (Niscola et al. 2007). Etoposide is excreted in saliva (0.5-4 µM) only at the short term (15 min) after high-dose administration (Mori et al. 2006).

Vincristine is isolated from Madagascar periwinkle (*Vinca rosea*) and used to treat solid tumors (mainly lung and breast), lymphomas and acute leukemia (Nussbaumer et al. 2011). Vincristine blocks mitosis by suppression of polymerization dynamics of microtubules, crucial in the process of chromosomal division to form new cells (Jordan 2002). Incidences of grade 3 and 4 oral mucositis vary from 4 to 10 % for regimens including vincristine for non-Hodgkin lymphoma (Jones et al. 2006, Keefe et al. 2007).

Cyclophosphamide is a nitrogen mustard with a broad treatment spectrum in solid tumors, lymphoma and leukemia (Nussbaumer et al. 2011). It is an alkylating agents able to covalently bind an alkyl group to DNA and to form inter- and intra-strand crosslinks, leading to cell apoptosis (Hall and Tilby 1992). Depending on the regimen, a risk of 0-27 % has been reported for grade 3 and 4 oral mucositis following treatment including cyclophosphamide (Sonis et al. 2004a). Salivary concentrations of cyclophosphamide range between 15-30 µM during the first 12 h after administration (Juma et al. 1979).

3. The role of the microbiota in mucositis

There is more and more interest in the role and/or the effect of the microbiome in mucositis. Current research recognizes and emphasizes their importance both for oral (Stringer and Logan 2015, Vanhoecke et al. 2015b, Vasconcelos et al. 2016) and gastrointestinal (van Vliet et al. 2010, Stringer 2013, Touchefeu et al. 2014) mucositis. Starting with the characteristics of the normal mucosa and its microbiome, we will further describe the microbial shifts upon chemotherapeutic treatment observed during observational studies of oral and gastrointestinal mucositis. A similar pathobiological model and underlying molecular pathways are used to describe both types of mucositis (section 1.3). Therefore, the interactions between the host and the microbiome and the mechanisms involved, will be described for alimentary mucositis in general in a last section.

3.1 Observational studies - Oral mucositis

3.1.1 Characteristics of the normal oral mucosa and its microbiome

The human oral mucosa consists of a stratified squamous epithelium on top of a connective tissue, or lamina propria (Squier and Kremer 2000, Vanhoecke et al. 2015b). Based

on its function and histology, there are three types of epithelium (Squier and Kremer 2000). The floor of the mouth and the buccal regions are covered with a non-keratinized epithelium. Together with the flexible connective tissue underneath, this forms the lining mucosa. In contrast, the masticatory mucosa consists of keratinized epithelium on top of a collagenous connective tissue and is therefore more resistant to mechanical forces associated with mastication on the gingiva and hard palate. The third type is the specialized epithelium, which covers the dorsum of the tongue and is a combination of keratinized and non-keratinized epithelium (Squier and Kremer 2000). All types of epithelium are covered with a 70-100 µm thick layer of salivary fluid consisting of water, mucins, salts, lipids, and proteins (Collins and Dawes 1987, Vanhoecke et al. 2015b). Next to the type of epithelium, saliva is a key factor influencing microbial composition as it provides buffering capacity and nutrient availability (Segata et al. 2012). Salivary mucins are high molecular weight glycoproteins and not only act as a primary food source for oral microbiota, but are also important as a host defense mechanism via formation of a physical-chemical barrier and for agglutination of microbiota removing them from the mouth by swallowing (Marsh and Martin 1999). Also other host factors can have an impact on the microbial composition, activity and stability, such as temperature, pH, redox potential, host genetics, and host defense molecules (lysozyme, lactoferrin, antimicrobial peptides, immunoglobulins, etc.) (Marsh and Martin 1999, Marsh and Devine 2011).

All these factors will contribute to a distinct microbial community in each region of the oral cavity: teeth, gingiva, tongue, cheek, lip, hard palate and soft palate (Aas et al. 2005, Dewhirst et al. 2010). In total, more than 700 species have already been described for the oral cavity (Aas et al. 2005, Zarco et al. 2012) and each individual is colonized by a unique microbiome (Wade 2013). The Human Microbiome Project characterized the oral communities from more than 200 healthy adults (Segata et al. 2012). Samples from different regions of the oral cavity were divided in three groups based on microbial community type: Group 1 contained buccal mucosa, keratinized gingiva, and hard palate; Group 2 contained saliva and tongue; and Group 3 contained sub- and supragingival plaque. At phylum level, Group 1 was dominated by Firmicutes (> 55 %), followed in decreasing order of relative abundance by Proteobacteria, Bacteroidetes and either Actinobacteria or Fusobacteria. Compared to Group 1, Group 2 had a lower abundance in Firmicutes and increased levels for Bacteroidetes, Fusobacteria, Actinobacteria and TM7 (*Candidatus* Saccharibacteria). The dental plaque samples in Group 3 consisted of an even lower level of Firmicutes and an increased level of Actinobacteria. On genus level, Group 1 was dominated by *Streptococcus*, whereas Group 2 and 3 had a more even distribution of *Streptococcus*, *Veillonella*, *Prevotella*, *Neisseria*, *Fusobacterium*, *Actinomyces* and *Leptotrichia* (> 2 % in Group 2 and 3) and *Corynebacterium, Capnocytophaga, Rothia* and *Porphyromonas* (> 2 % in Group 3). Group 1 showed a lower

diversity (measured by the inverse Simpson index), probably due to the high abundance of *Streptococcus* (Segata et al. 2012).

Oral microbiota are present as planktonic cells in saliva or as biofilms on both dental and mucosal surfaces (Avila et al. 2009, Vanhoecke et al. 2015b). The early colonizers of the biofilms are *Streptococcus* and *Actinomyces* as they can adhere to the oral surfaces. The immature biofilm consist of 80 % *Streptococcus* spp., which forms the basis for a multi-layered multi-species biofilm (Kolenbrander et al. 2010). One of the key species in biofilm formation is *Fusobacterium* as it creates anaerobic microenvironments and makes physical bridges (Kolenbrander et al. 2010). This 'bridging' capacity is established by special cell surface proteins (adhesins) providing inter-species adherence (Kaplan et al. 2009). In contrast to multilayer dental plaques, mucosal biofilms are less characterized. The high turnover rate of the epithelial cells makes it more difficult to adhere which leads to the formation of one single biofilm layer and a wash-out of non-adhered microbiota by saliva (Vanhoecke et al. 2015b).

Certain genera, such as *Porphyromonas*, *Treponema*, and *Tannerella,* are characterized by pathogenic members and therefore associated with disease. However, it has been shown that also in the healthy oral cavity, these genera are present at low abundance (Segata et al. 2012). Moreover, also commensal species can become pathogenic in response to certain triggers in the oral cavity such as changes in oral hygiene (Avila et al. 2009). However, oral infections are most likely caused by consortia of biofilm microbiota rather than a single pathogenic species (Jenkinson and Lamont 2005).

3.1.2 Oral microbial shifts upon chemotherapeutic treatment

Although the role of the oral microbiota in chemotherapy-induced mucositis is still unclear, there is growing interest in this topic (Stringer and Logan 2015, Vanhoecke et al. 2015b, Vasconcelos et al. 2016). In 2007, a first review stated that changes in the oral microbiome are inconsistent during chemotherapy, due to the great variability in patient populations, sample types, and sample collection methods (Napenas et al. 2007). Nevertheless, both this and later studies most frequently isolated *Enterobacteriaceae* spp., *Pseudomonas* spp., *Klebsiella pneumonia* and *E. coli* as Gram-negative species in blood and oral swabs during chemotherapy/HSCT (Napenas et al. 2007, Panghal et al. 2012). Similarly, the most commonly isolated Gram-positive species were *Staphylococcus* spp. and *Streptococcus* spp. (Napenas et al. 2007, Napenas et al. 2010, Olczak-Kowalczyk et al. 2012, Panghal et al. 2012). Pediatric studies showed that the *Streptococcus oralis* group of viridans streptococci is persistent following chemotherapy and total body irradiation (Lucas et al. 1997) and that the presence of *Staphylococcus* spp. and *Enterococcus* spp. is correlated with oral lesions (Olczak-Kowalczyk et al. 2012). Also other microorganisms have been shown to be associated with mucositis or oral ulcerations. In HSCT patients, *Porphyromonas gingivalis* in

particular, but also *Parvimonas micra*, *Treponema denticola* and *Fusobacterium nucleatum* were associated with oral ulcerations (Laheij et al. 2012). Fungal species, such as *Candida,* (de Mendonca et al. 2012, Laheij et al. 2012, Olczak-Kowalczyk et al. 2012) and viruses, such as *Herpes simplex virus* (HSV)-1, (de Mendonca et al. 2012, de Mendonca et al. 2015) are also associated with mucositis and/or oral lesions.

In the past, Gram-negative bacteria were the main cause of infections in neutropenic cancer patients. However, a shift towards Gram-positive infections has been shown in the 1990s (Zinner 1999) and also more recent studies have indicated Gram-positive bacteria as major pathogens causing infections (Panghal et al. 2012). This can be explained by the prophylactic use of fluoroquinolones, the use of high-dose chemotherapy causing oral mucosal barrier disruption and the use of central venous lines increasing the risk of infections with skin bacteria (Zinner 1999, Panghal et al. 2012). Viridans streptococci are one of the main Gram-positive pathogens causing infections. Factors that predispose to the development of viridans streptococci sepsis are neutropenia, oral mucositis, high-dose cytarabine and antimicrobial prophylaxis with trimethoprim-sulphamethoxazole or quinolone (Shenep 2000, Tunkel and Sepkowitz 2002).

Most of the previous mentioned studies used cultivation techniques to identify and quantify the oral microbiota. Recently, molecular techniques are being used to study the microbiome, leading to new in-depth insights. The use of 16S rRNA clone libraries of oral buccal samples of 9 breast cancer patients before and after chemotherapy resulted in species previously unidentified in patients. Moreover, a shift to a more complex microbial community after chemotherapy was observed with 60 % of the species only present after the treatment (Napenas et al. 2010). In pediatric patients, 454-sequencing of oral mucosal bacterial samples showed a lower diversity and higher inter-individual variability for patients compared to reference individuals. Furthermore, they found a higher microbial diversity before the start of chemotherapy and a more significant modification of the bacterial community by chemotherapy (before the occurrence of mucositis) in patients who later developed oral mucositis (Ye et al. 2013). In a recent study with pediatric patients with acute myeloid leukemia (AML), amplicon sequencing of oral and stool microbiota showed a correlation between increased variability of the microbial diversity over time with higher risk of infection, respectively during an after chemotherapy (Galloway-Pena et al. 2017).

In conclusion, only limited studies dealing with the effect of chemotherapy on the oral microbiome were conducted so far and most of them used cultivation techniques. Moreover, research on the composition of the microbiota and/or relative abundance of different species before and after chemotherapy is limited, making it difficult to clearly indicate the shifts occurring after chemotherapy or linked with mucositis (Vanhoecke et al. 2015b). These shifts also widely vary depending on the chemotherapy regimen, sampling- and patient-related

variables (Vanhoecke and Stringer 2015). More longitudinal clinical investigations are needed to fully understand the interactions between the host, oral mucositis and the oral microbiome (Vasconcelos et al. 2016).

3.2 Observational studies - Gastrointestinal mucositis

3.2.1 Characteristics of the normal gastrointestinal mucosa and its microbiome

The human digestive tract is partitioned in different sections, each with its own mucosa and microbiome. In this dissertation the focus will be on the large intestine, as our research is investigating the effect of chemotherapy on the colon microbiome. The mucosa of the large intestine has a single layered epithelium which is covered with a protectant mucus-layer. Goblet cells in the epithelium will produce mucins, which are the main building blocks of the mucus-layer. In contrast with the small intestine, the colon has a two-layered mucus system, both built by mucin 2 (MUC2). The inner dense layer is attached to the epithelial cells, whereas the outer layer is loose and unattached (Johansson et al. 2011, Johansson et al. 2013). The major functions of the mucosa are the absorption of nutrients and barrier formation (Van der Flier and Clevers 2009). Together with the mucus layer, tight junctions and the innate and adaptive immune system will establish this mucosal barrier protecting against gut microbial invasion (Van den Abbeele et al. 2011).

Not only the mucosa, but also the microbiota differ in each part of the gastrointestinal tract, depending on the pH, retention time, exposure to oxygen, substrate availability and host secretions (Macfarlane and Macfarlane 1997, Flint et al. 2012). The colon harbors the most dense and complex microbial community (Walter and Ley 2011) containing hundreds of different species, mostly member of the five most abundant bacterial phyla (Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria and Verrucomicrobia) (Walter and Ley 2011). Next to longitudinal variation, axial variation occurs in the gastrointestinal tract, with different microbiota in the lumen compared to the mucus layer. A gradient in increasing microbial diversity and density is observed from the mucosa to the lumen (Van den Abbeele et al. 2011). A clear difference in mucus-associated microbiota was seen with higher levels of Firmicutes and more specifically Lachnospiraceae and Ruminococcaceae (Nava et al. 2011).

Within one individual, the intestinal microbial community is relatively stable over time, although several factors can cause significant changes, such as antibiotics and diet, but also chemotherapy. In contrast, large inter-individual differences exist in abundance and composition of the stool microbiome, whereas functional profiles remain more stable over different individuals (Huttenhower et al. 2012). Both adverse and beneficial functions are exerted by the human gut microbiome. Adverse functions include infection and production of toxins (Louis et al. 2014), whereas beneficial functions can be protective, structural or

metabolic, comprising for example short-chain fatty acid (SCFA) production, vitamin production, immune system development and mucosal barrier fortification (O'Hara and Shanahan 2006). The major metabolic pathways that drive the growth of colon microbiota are carbohydrate fermentation (with production of SCFA and gases) (Falony and De Vuyst 2009), protein fermentation (with production of toxic and carcinogenic products) (Louis et al. 2014) and mucus fermentation (with production of SCFA and oligosaccharides) (Belzer and De Vos 2012).

3.2.2 Gastrointestinal microbial shifts upon chemotherapeutic treatment

A lot of research on gastrointestinal mucositis was conducted using laboratory animals. Although there are differences between inflammatory diseases in humans and animals and their respective microbiome, these models are important to provide insights in the pathobiology of mucositis (Ley et al. 2005, Touchefeu et al. 2014). Tests with germ-free mice treated with irinotecan have confirmed that microbiota modify the mucositis process, as conventional mice had higher inflammatory markers, more lesions and higher intestinal permeability in comparison with germ-free mice (Pedroso et al. 2015). Multiple studies have shown shifts in the microbiome following treatment with 5-FU, irinotecan and MTX. In a rat study, 5-FU caused a shift in composition from predominantly Gram-positive to Gram-negative bacteria, as shown by microbiological culture techniques (von Bultzingslowen et al. 2003). A later study with 5-FU showed a decrease in *Enterococcus* spp., *Lactobacillus* spp. and *Streptococcus* spp. in the colon using standard microbiological culture techniques and an increase in *Clostridium* spp. and *Staphylococcus* spp. in fecal samples at 24 h using q-PCR (Stringer et al. 2009c). Similar rat studies have been done with irinotecan, which reported an increase in *E. coli* and a decrease in *Bifidobacterium* spp. and *Lactobacillus* spp. (Stringer et al. 2007, Stringer et al. 2008, Stringer et al. 2009a). An increase in Clostridium cluster XI and Enterobacteriaceae was seen for both irinotecan and irinotecan/5-FU treatment regimens in rats (Lin et al. 2012). Also MTX impacts the microbiota in rats, decreasing the number and diversity of microbiota (Fijlstra et al. 2015). In general, a shift from commensal microbiota (*Bifidobacterium* spp. and *Lactobacillus* spp.) to *Escherichia* spp., *Clostridium* spp. and *Enterococcus* spp. has been observed after chemotherapeutic treatment in animal studies (Touchefeu et al. 2014). These microbiota are also frequently isolated in the blood of cancer patients, causing bacteremia (Montassier et al. 2013).

Human studies have also reported changes in the fecal microbiota following chemotherapeutic treatment. In pediatric patients, a decrease in total number and diversity of microbiota was reported during chemotherapy regimens. Fluorescent In Situ Hybridization (FISH) showed a decrease in anaerobic bacteria (*Bacteroides* spp., *Clostridium* cluster XIVa, *Faecalibacterium prausnitzii*, and *Bifidobacterium* spp.) combined with an increase in

potentially pathogenic aerobic *Enterococcus* spp. The authors concluded that the disturbed balance could increase the risk for Gram-positive aerobic infections among immune-compromised patients with cancer (van Vliet et al. 2009). Another study showed a decrease in the *Clostridium* cluster IV and XIXa, *Bifidobacterium*, *Lactobacillus*, *Veillonella,* and *Faecalibacterium prausnitzii* in patients receiving different chemotherapy regimens. This disturbed balance favored colonization with *Clostridium difficile* and *Enterococcus faecium* (Zwielehner et al. 2011). After conditioning chemotherapy for bone marrow transplantation, a sharp reduction in alpha diversity of the fecal microbiota and a drastic drop in *Faecalibacterium* accompanied by an increase of *Escherichia* was observed (Montassier et al. 2014). A similar study showed a decrease in abundances of Firmicutes and Actinobacteria and an increase in Proteobacteria in fecal samples of patients following chemotherapy (Montassier et al. 2015). Some changes can also be associated with chemotherapy-induced diarrhea. Stringer et al. (2013) reported decreases in *Lactobacillus* spp., *Bifidobacterium* spp., *Bacteroides* spp., *Enterococcus* spp. and methanogenic archaea and an increase in *Escherichia coli* and *Staphylococcus* spp. in fecal samples of patients with chemotherapy-induced diarrhea. In general, the most frequent changes in patients receiving chemotherapy are a decrease in *Bifidobacterium*, *Clostridium* cluster XIVa, *Faecalibacterium prausnitzii* and an increase in *Enterobacteriaceae* and *Bacteroides* (Touchefeu et al. 2014).

In conclusion, both animal and clinical studies indicate that chemotherapeutic treatment causes major modifications to the gut microbiome and can lead to a disturbed balance. This imbalance will possibly also contribute to the mucositis process (Touchefeu et al. 2014).

3.3 Interactions between the microbiome and the mucosa in the context of mucositis

The interactions between the microbiome and the host mucosa are similar in oral and gastrointestinal mucositis, as they are both described by the same pathobiological model and as similar processes occur in both regions (Sonis 2004). Therefore, the mechanisms underlying host-microbe cross talk during mucositis will be relevant for both types of mucositis.

3.3.1 Mucosal barrier function

A healthy gut is characterized by an intestinal homeostasis and the commensal microbiota can help the intestinal mucosa in maintaining this balance via different mechanisms (Figure 1.1) (van Vliet et al. 2010, Touchefeu et al. 2014). The integrity of the mucosal barrier is crucial in this homeostasis and is ensured by both the mucus layer and the tight junction formation in the epithelial barrier. Dysregulation of these interactions can lead to inflammatory

bowel disease (Medzhitov 2007) or to mucositis after dysregulation of the mucosal barrier by chemo- or radiotherapy (Touchefeu et al. 2014).

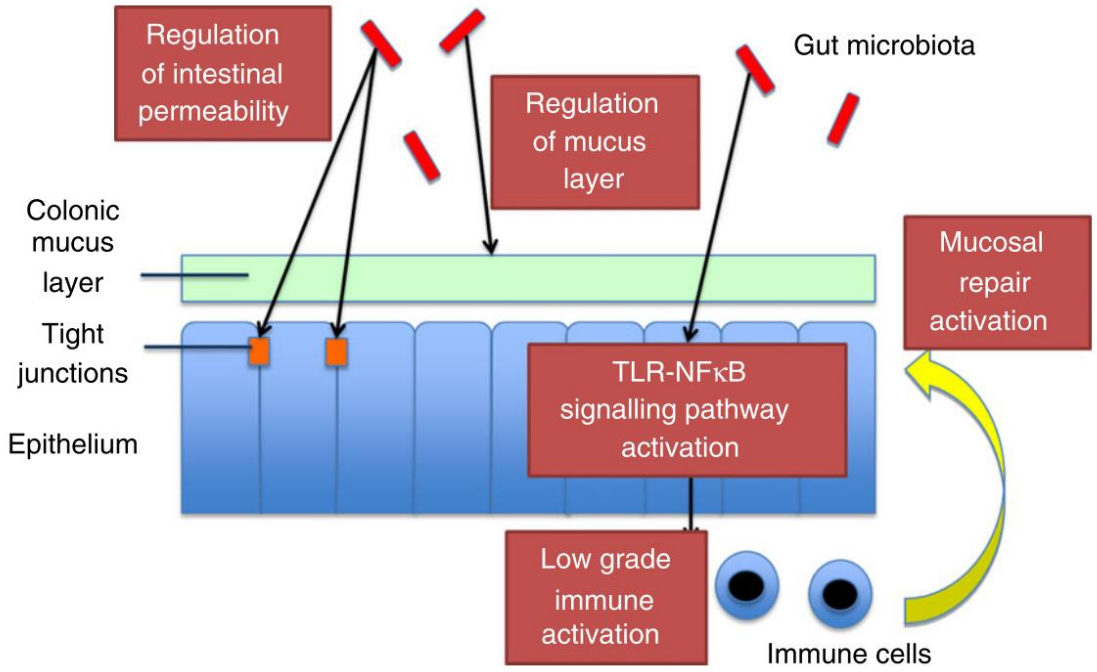


Figure 1.1 - Protective actions of intestinal microbiota on the intestinal mucosa (Touchefeu et al. 2014).

The first crucial step in maintaining the mucosal barrier is reducing **epithelial permeability**. However, both human and rat studies have shown that chemotherapy increases epithelial permeability (Keefe et al. 1997, Carneiro-Filho et al. 2004, Russo et al. 2013). Epithelial integrity and tight junctions are the main key factors to assure low permeability and chemotherapy can impact both of them. In the third phase of the pathobiology of mucositis, epithelial cell loss will aggravate the permeability in the alimentary tract due to activation of apoptosis via different pathways (Sonis 2007). Moreover, chemotherapy has also been shown to disrupt tight junctions, both in the oral and gastrointestinal mucosa (Hamada et al. 2010, Wardill et al. 2016). In contrast, commensal gut microbiota can enhance barrier function by influencing expression and distribution of tight junctions (Ulluwishewa et al. 2011). Furthermore, microbiota can induce the production of heat shock proteins which preserve the viability of epithelial cells in stress conditions and maintain the epithelial integrity (Arvans et al. 2005, Matsuo et al. 2009). Also microbial metabolites can attenuate epithelial permeability. Butyrate, a short chain fatty acid produced by gut microbiota, has shown to reduce intestinal permeability in 5-FU induced mucositis in mice (Ferreira et al. 2012). This indicates that shifts in microbiota could play a role in epithelial permeability, influencing the severity of mucositis in the ulcerative phase (van Vliet et al. 2010).

Next to epithelial integrity, the mucosal barrier is maintained by the **mucus layer**. Both the composition of the mucus layer and the mucus secretion are affected by chemotherapy in rat studies (Stringer et al. 2009a, Stringer et al. 2009b, Stringer et al. 2009c). Goblet cells (specialized epithelial cells in production of mucin) decreased in the colon following irinotecan treatment, whereas mucin secretion increased. This is linked with higher abundance of cavitated goblet cells (cells which have released mucins by exocytosis) (Stringer et al. 2009a, Stringer et al. 2009b). Similar trends were seen following treatment with 5-FU. Total goblet cell numbers and abundance of cavitated goblet cells increased in the jejunum (Stringer et al. 2009c). Similarly, jejunal mucin content was reduced on day 1 and 3, but increased again at day 7 after 5-FU treatment (Saegusa et al. 2008). However, in methotrexate-induced mucositis, goblet cells were only decreased in the small intestinal crypts and not in the villi (Verburg et al. 2000). Not only chemotherapy, but also gut microbiota can affect goblet cells and the mucus layer. Directly, via the local release of bioactive factors or indirectly, via activation of host immune cells (Deplancke and Gaskins 2001). Germfree mice are shown to have less and smaller goblet cells and a different mucus composition in comparison with conventional mice (Kandori et al. 1996, Deplancke and Gaskins 2001). The enhancement of mucus secretion in the presence of microbiota is explained by the advantages it offers for the gut microbiota, as it serves as an energy source and protects the microbiota against rapid luminal expulsion by offering a surface to adhere (Deplancke and Gaskins 2001). Microbiota also steer the composition of the mucins by regulating genes encoding mucins, such as the upregulation of *muc-2* and *muc-3* genes by certain *Lactobacillus* strains (van Vliet et al. 2010). Last, also host-derived inflammatory mediators, such as IL-1, IL-4, IL-6, IL-9, and TNF-α have been shown to increase mucus secretion (Deplancke and Gaskins 2001). In conclusion, multiple key factors in the mucositis process such as chemotherapy, microbiota and inflammatory proteins, can impact the mucus layer improving or attenuating the strength of the mucosal barrier.

3.3.2 Host-microbe communication

Normal host-microbe communication

Epithelial cells are key players in the communication between the host and its microbiota. Their **pattern recognition receptors (PRRs)** are able to recognize and bind microbe associated molecular patterns (MAMPs). These are microbial macromolecular ligands, such as lipopolysaccharide, flagellin, peptidoglycans or formylated peptides (Medzhitov 2007, Neish 2009). These MAMPs are produced by both pathogenic and commensal microbiota, however, PRR do not themselves distinguish between pathogens and commensals (Rakoff-Nahoum et al. 2004, Medzhitov 2007). PRR can be transmembranic, like Toll-like receptors (TLRs),

intracellular, like Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), or secreted, like collectins and are present in all multicellular organisms (Medzhitov 2007, Neish 2009). TLR activation by binding of a MAMP, triggers a cascade of cellular signals, activating the NF-κB pathway and ultimately developing an inflammatory response with the upregulation of pro-inflammatory cytokines and chemokines (Doyle and O'Neill 2006, van Vliet et al. 2010, Vanhoecke et al. 2015b). These chemoattractant cytokines can further attract immune cells and also have antimicrobial properties (Durr and Peschel 2002, Kagnoff 2006). This entire process forms the basis of the innate immune response, which establishes an intestinal homeostasis leading to a low grade inflammation, barrier formation, wound repair and tissue regeneration (Medzhitov 2007, Cario 2008, Touchefeu et al. 2014) (Figure 1.1).

Interference of chemotherapy and microbiota with host-microbe communication

A highly important pathway in mucositis and in innate immunity is the **TLR/NF-κB signaling**. Cancer therapy will induce cell death which will lead to the release of DAMPs. By binding to TLRs, these will activate an inflammatory response (Vasconcelos et al. 2016). However, when mucosal injury starts and permeability increases, the abundance of MAMPs will increase, further stimulating the overall immune status of the tissue (Vanhoecke and Stringer 2015, Vasconcelos et al. 2016). MAMPs known for oral diseases are, for example, *Treponema denticola* flagellin (Beklen et al. 2009) and *Porphyromonas gingivalis* LPS (Kocgozlu et al. 2009). Interestingly, both species were also associated with oral ulcerations after high-dose chemotherapy (Laheij et al. 2012).

Both DAMPs and MAMPs will bind to **TLRs**, which have been shown to be crucial in host-microbe cross talk and epithelial homeostasis (Rakoff-Nahoum et al. 2004). The disturbance of the host-microbe interactions by chemotherapy is (partially) mediated by TLR signaling (Vanhoecke and Stringer 2015). In particular TLR4, which binds to lipopolysaccharides (LPS), is linked with chemotherapy-induced gut toxicity (Wardill et al. 2014). MTX-treated rats showed an increase in TLR4 protein in the small intestine during gut toxicity (Hamada et al. 2013). In contrast, irinotecan caused a decrease in both TLR4 and TLR5 in rats and the absence of both is correlated with a better wound healing capacity (Gibson et al. 2016). Moreover, the shifts in microbiota following chemotherapy (see above) will lead to changes in available ligands to bind to TLRs and ultimately cause an altered TLR activity (Stringer and Logan 2015).

A cascade of signals will further lead to the activation of **NF-κB**, which is a key molecule in the pathobiology of mucositis (Sonis 2004, Sonis 2007, Vanhoecke and Stringer 2015). NF-κB is not only activated by TLRs, but also by chemotherapy directly or indirectly via ROS (Sonis 2004). Rat studies showed increased tissue and serum levels of NF-κB following chemotherapy (Logan et al. 2008a, Logan et al. 2008b). Also *in vivo,* increased levels of NF-

κB are shown following cytotoxic chemotherapy in oral mucosa biopsies (Logan et al. 2007). Commensal microbiota, such as *Bacteroides thetaiotaomicron*, *Bifidobacterium infantis* and *Lactobacillus salivarius* are able to decrease NF-κB levels (Kelly et al. 2004, O'hara et al. 2006). Therefore, regulation of the microbiota following chemotherapy can reduce NF-κB production and further inflammatory processes (Stringer 2013).

Next to TLR-NF-κB signaling, **MAPK signaling** is a major pathway in mucositis and can also be activated by bacterial ligands (Figure 1.2) (Sonis 2007, Stringer and Logan 2015). MAPK signaling is involved in the regulation of multiple eukaryotic cell activities including activation of transcription factors, stress response, differentiation, and growth (Handfield et al. 2008). Activation of MAPK signaling by bacterial ligands will lead to production of pro-inflammatory cytokines and apoptosis (Stringer and Logan 2015). Moreover, rat studies showed that MAPK signaling was upregulated in the intestinal tract following irinotecan treatment (Bowen et al. 2007a, Bowen et al. 2007b).

Also the **inflammasome** is involved in host-microbe communication. These protein complexes are formed in the cytosol after sensing MAMPs or DAMPs and are mostly formed of NLRs. They regulate the activation of caspase-1, leading to cleavage of IL-1 to their bioactive forms IL-1β and IL-18, which are pro-inflammatory cytokines (Guo 2015). It has been shown in mice that inflammasomes can mediate gastrointestinal mucositis via IL-1β and IL-18 and that they are ROS dependent (Arifa 2014).

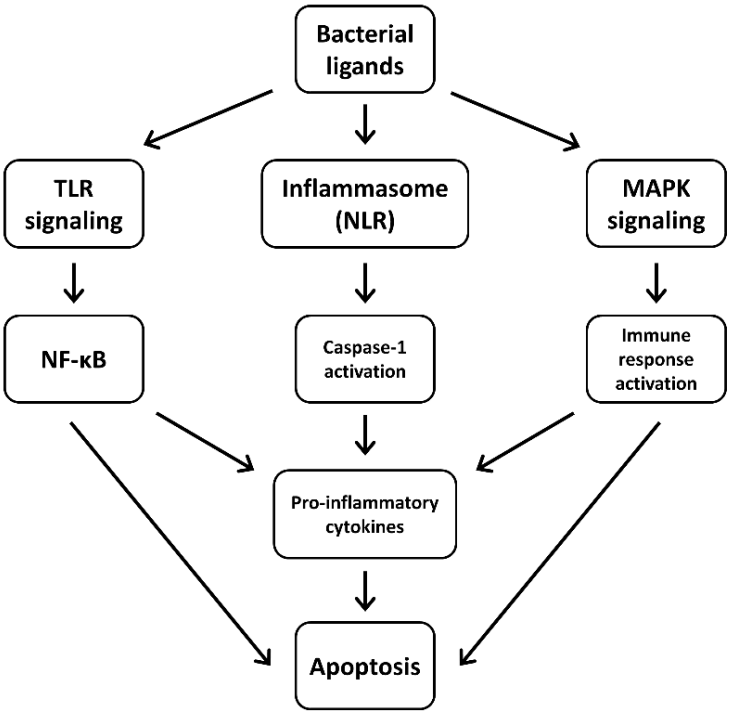


Figure 1.2 - Microbiota can influence mucositis by interfering with TLR/NF-κB pathway, MAPK signaling and the inflammasome (based on Stringer and Logan (2015)).

Both NF-κB, MAPK signaling and the inflammasome will lead to the activation of **pro-inflammatory cytokines** such as TNF, IL-1β and IL-6, which are all important in the pathobiology of mucositis (Sonis 2004, Stringer and Logan 2015). These pro-inflammatory cytokines have all been shown to be upregulated following irinotecan, 5-FU and MTX treatment in rats (Logan et al. 2008a, Logan et al. 2009).

All these processes will lead to an **inflammatory status and immune activation** in the tissue (van Vliet et al. 2010). Commensal microbiota provide a continuous exposure to bacterial ligands, which can bind to TLR and lead to a low grade inflammatory status (Rakoff-Nahoum et al. 2004). This homeostasis is also regulated by immune effector molecules, such as Immunoglobulin A (van Vliet et al. 2010). Immunoglobulin A is the most abundant antibody in all mucosal secretions and is produced by B-cells in the gut-associated lymphoid tissue (GALT) (Fagarasan and Honjo 2003). By regulating this immune effector molecules, both microbiota and their products are able to maintain homeostasis (van Vliet et al. 2010).

3.3.3 Mucosal repair and wound healing

The pathobiological model of mucositis shows that wound healing is crucial in the recovery from mucositis (Sonis 2004). The epithelial wound healing process exists of two mechanisms: migration and proliferation. Migration is the most important process at the edge of the wound, whereas proliferation is taking place at more distinct places (Zahm et al. 1997). Next to epithelial cells also fibroblasts are important in the wound healing process as they produce the extracellular matrix (Ryu et al. 2009). Fibroblasts and vascular endothelium produce cyclooxygenase-2 (COX-2), which stimulates angiogenesis leading to submucosal restructuring (Sonis 2007). Moreover, epithelial migration, proliferation and differentiation will be steered from the submucosa during the healing phase of mucositis (Sonis 2004, Sonis 2007). Although a spontaneous healing is occurring after cessation of the cancer treatment, the structure of the submucosa will never be identical to its initial state, even after complete epithelial closure (Sonis 2007) (Denham and Hauer-Jensen 2002).

Radiotherapy may disturb the normal wound healing process by interfering with the vascular system, fibroblasts and growth factor levels (Tibbs 1997). However, the use of recombinant human epidermal growth factor has been shown to increase proliferation of fibroblasts and epithelial cells leading to faster wound healing capacity in radiotherapy-induced oral mucositis in rodents (Lee et al. 2007, Ryu et al. 2009). Although studies are limited, there are some indications that also chemotherapy may impact wound healing capacity (Bland et al. 1984).

More importantly, the microbiome has been shown to majorly impact wound repair and tissue regeneration (Cario 2008, Touchefeu et al. 2014) (Figure 1.1) and protect the gut against

injuries (Rakoff-Nahoum et al. 2004). The positive effect of microbiota on wound healing was confirmed by tests with germ-free animals. In the absence of microbiota, lower rates of epithelial cell migration and cell turnover were observed (Rolls et al. 1978). Moreover, the migration of the cells to the top of the villi was retarded (Savage et al. 1981). Not only intestinal wound healing, but also repair of skin incisions has been shown to be enhanced in the presence of normal intestinal microbiota in comparison to germ-free mice (Okada 1994).

However, microbiota can also negatively affect wound healing. Edwards and Harding (2004) showed that low bacterial levels improve wound healing in chronic wounds, whereas infection with a high bacterial load are detrimental for the wound healing process. The importance of microbial concentration in the wound healing process was also confirmed *in vitro* (De Ryck et al. 2015). Next to microbial load, also the composition of the microbiome is important, as infection with certain species may delay wound healing and others may even have a beneficial impact on wound healing (Laheij et al. 2013, De Ryck et al. 2015). De Ryck et al. (2015) showed that *S. mitis* and *S. oralis* significantly improved wound healing capacity, whereas *Klebsiella oxytoca* and *Lactobacillus salivarius* reduced healing of oral epithelial cells in an *in vitro* model. Also *Porphyromonas gingivalis* has been shown to reduce wound healing and is linked with oral ulcerations during mucositis (Laheij et al. 2013). In contrast, *Akkermansia muciniphila* is a wound-mucosa-associated species of the human gut which can stimulate both migration and proliferation of enterocytes (Alam et al. 2016). Not only microbial cells itself, but also bacterial metabolites may impact wound healing. For example, the supernatant of *Lactobacillus* cultures has been shown to reduce wound closure (Halper et al. 2003). The effect of bacterial LPS on wound healing depends on its concentration, with low concentrations causing an improvement in wound healing by activation of epidermal growth factor receptor, whereas high concentrations cause a reduction in healing capacity (Koff et al. 2006). Butyrate has been shown to stimulate migration of cells *in vitro*, leading to a better repair of mucosal damage (Wilson and Gibson 1997, Hamer et al. 2008). So far it is not clear which mechanisms are steering the impact of microbiota on wound healing. However, quorum sensing molecules are thought to play a role in signaling during wound healing (De Ryck et al. 2015).

Next to microbiota and their metabolites, also host-derived products can impact wound healing capacity. In saliva, histatin 1 and histatin 2 are identified as the most important molecules stimulating wound closure in the oral cavity (Oudhoff et al. 2008). Also MMPs, zinc-dependent endopeptidases crucial in maintaining homeostasis in the extracellular matrix, may be important in both wound healing and mucositis. MMPs are shown to be increased following chemotherapeutic treatment both in animal studies (Al-Dasooqi et al. 2010) and clinical studies (Stringer et al. 2013). Moreover, in the pathobiology of mucositis they are responsible for the degradation of the extracellular matrix (Sonis 2004). With regards to wound

healing, MMP-9 is linked with accelerated healing, whereas MMP-2 reduced wound healing capacity (de Bentzmann et al. 2000).

Some of the current treatments for mucositis also focus on stimulating wound healing capacity of the epithelium. For example, the use of LLLT, which is recommended for the prevention of oral mucositis in patients receiving HSCT conditioning high-dose chemotherapy (Lalla et al. 2014). It is hypothesized that the effectiveness of LLLT is based on the fact that the laser light is absorbed by chromophores in the mitochondria which will activate ATP production, cell proliferation, protein synthesis and stimulate tissue repair (Karu 1988, Simoes et al. 2009).

3.3.4 Conclusion

In conclusion, both chemotherapy and microbiota interfere with multiple crucial processes and signaling pathways during mucositis: the integrity of the components of the mucosal barrier, the underlying immune system and signaling mechanisms such as TLR-NF-κB and MAPK pathways. This interference will ultimately alter the inflammatory and immune status of the epithelium and repair mechanisms. In a healthy individual, interactions between all these processes result in a state of homeostasis. However, cancer treatment will disturb this balance and the microbiota of the alimentary tract can both help to restore this balance or further aggravate the dysbiosis, depending on different factors such as bacterial concentration, bacterial composition and host factors.

4. Research questions

Both oral and gastrointestinal mucositis are prevalent side effects of chemotherapeutic treatments which majorly affect patient's quality of life. More and more, mucositis research is focusing on the role of microbiota in the development of both oral and gastrointestinal mucositis. As chemotherapeutic agents are present in saliva an feces and are theoretically able to cause damage to the DNA of the microbiota, chemotherapy might not only damage the host cells, but also the host microbiome. *In vivo* studies have indeed reported microbial shifts following chemotherapeutic treatment, both in rats and in humans. This disturbed microbiome might further aggravate the mucositis development. However, it is still unclear if the chemotherapy disturbs the microbiota directly or if the chemotherapy disturbs the host environment and thereby creates a dysbiotic microbiome.

To further answer this question, two different approaches were used during this PhD thesis (Figure 1.3). In the first two chapters, we focused on the direct effect of chemotherapy on both oral and gastrointestinal microbiota. In chapter 4 and 5, the addition of a host compartment made it possible to investigate interactions between chemotherapy, microbiota and the host. 5-FU and irinotecan were chosen as chemotherapeutic agents in this thesis, as previous *in vivo* studies have shown that both 5-FU and irinotecan have an impact on the host microbiota (see section 3.2.2).

5-FU is a commonly used chemotherapeutic agent characterized by high incidences of mucositis. To evaluate the direct effect of 5-FU on different oral microorganisms, an *in vitro* study with monocultures was performed in **Chapter 2**. The effect of physiologically relevant concentrations of 5-FU (0.1-50 µM) on growth and viability of 11 oral microorganisms was assessed with growth curves and flow cytometry. Moreover, the possible role of DPD, an enzyme involved in 5-FU resistance, was investigated.

Next to 5-FU, irinotecan also highly induces gastrointestinal mucositis. To assess the direct impact of 5-FU and SN-38 (the active metabolite of irinotecan) on the gut microbiome, an *in vitro* colon simulation with microbiota from different healthy individuals was performed **(Chapter 3)**. Changes in microbial functionality and community composition were monitored following multiple treatments in a Mucosal-Simulator of the Human Intestinal Ecosystem (M-SHIME®).

In **Chapter 4**, an *in vitro* oral co-culture model was used to study the interactions between 1) the chemotherapeutic agents 5-FU, 2) an oral microbial biofilm, and 3) an oral epithelial monolayer. The impact of different microbial sample types (saliva, buccal or tongue) and donor types (healthy individuals or patients suffering from mucositis) on wound healing was reported and linked with bacterial count and composition.

Finally, a longitudinal observational *in vivo* study studied the oral microbiota of pediatric patients treated for hematological malignancies **(Chapter 5)**. Analysis of the microbiome before, during and after chemotherapy was performed, including periods with and without mucositis. The microbial shifts were reported and correlated with clinical data such as chemotherapy, mucositis, antibiotic treatment, neutropenia, inflammation, use of mouth rinses, and pain.

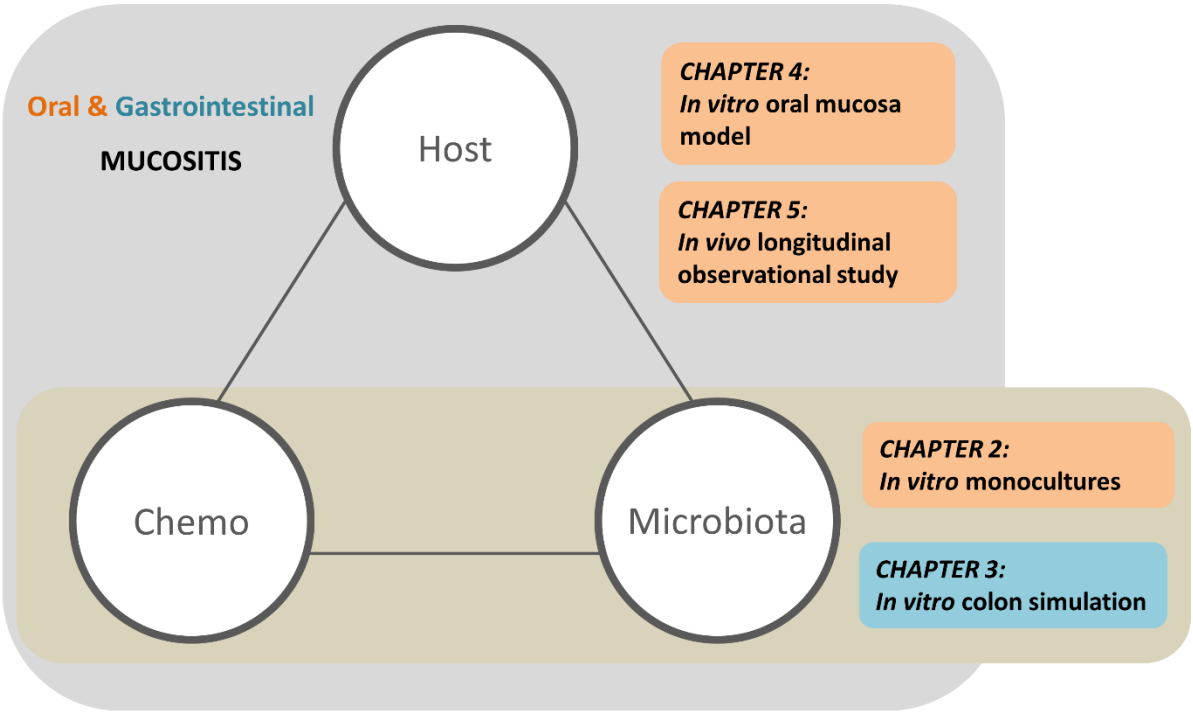


Figure 1.3 - Overview of the experimental chapters in this PhD thesis.

CHAPTER 2

5-Fluorouracil sensitivity varies among oral microorganisms

This chapter has been redrafted after

Vanlancker, E., B. Vanhoecke, R. Smet, R. Props and T. Van de Wiele (2016). 5-Flourouracil sensitivity varies among oral microorganisms. *Journal of Medical Microbiology,* **65**, 775-783.

CHAPTER 2

5-Fluorouracil sensitivity varies among oral microorganisms

Abstract

5-Fluorouracil (5-FU), a commonly used chemotherapeutic agent, often causes oral mucositis, an inflammation and ulceration of the oral mucosa. Microorganisms in the oral cavity are thought to play an important role in the aggravation and severity of mucositis, but the mechanisms behind this remain unclear. Although 5-FU has been shown to elicit antibacterial effects at high concentrations (>100 µM), its antibacterial effect at physiologically relevant concentrations in the oral cavity is unknown. This study reports the effect of different concentrations of 5-FU (range 0.1-50 µM) on the growth and viability of bacterial monocultures that are present in the oral cavity and the possible role in the activity of dihydropyrimidine dehydrogenase (DPD), an enzyme involved in 5-FU resistance. Our data showed a differential sensitivity among the tested oral species towards physiological concentrations of 5-FU. *Klebsiella* oxytoca, *Streptococcus salivarius*, *Streptococcus mitis*, *Streptococcus oralis*, *Pseudomonas aeruginosa* and *Lactobacillus salivarius* appeared to be highly resistant to all tested concentrations. In contrast, *Lactobacillus oris*, *Lactobacillus plantarum*, *Streptococcus pyogenes*, *Fusobacterium nucleatum* and *Neisseria mucosa* showed a significant reduction in growth and viability already starting from very low concentrations (0.2 - 3.1 µM). We could also provide evidence that DPD is not involved in the 5-FU resistance of the selected species. The observed variability in response to physiological 5-FU concentrations may explain why certain microbiota lead to a community dysbiosis and/or an overgrowth of certain resistant microorganisms in the oral cavity following cancer treatment.

1. Introduction

5-FU is a commonly used chemotherapeutic agent for colon and breast cancer. It is an anti-metabolite that inhibits human thymidylate synthase and is incorporated in DNA and RNA (Grem 2000, Longley et al. 2003). One of the main side effects of 5-FU treatment is oral mucositis, an inflammation and ulceration of the mucosa of the oral cavity (Peterson and Sonis 1982, Carnel et al. 1990). It majorly affects the quality of life of patients, including problems with eating, speaking and drinking, and treatment mainly is concentrated on pain relief and oral hygiene (Villa and Sonis 2015).

Along the gastrointestinal tract, microorganisms play an important role in sustaining homeostasis and health (Aziz et al. 2013). Evidence is also emerging that microorganisms are involved in the development and severity of 5-FU-induced mucositis (Stringer and Logan 2015, Vanhoecke et al. 2015b). Changes in the composition of the oral microorganisms have been reported for chemotherapy in human studies (Lucas et al. 1997, Napenas et al. 2010) and for 5-FU specifically in a rat study (von Bultzingslowen et al. 2003), with a shift towards more Gram-negative rods and bacterial translocation to cervical and mesenteric lymph nodes following treatment with 5-FU. A number of studies have been looking at the antibacterial effect of 5-FU on monocultures of mainly pathogenic strains such as *Pseudomonas*, *Klebsiella* and *Staphylococcus* (Wright and Matsen 1980, Ueda et al. 1983, Bodet et al. 1985, Takahata et al. 1986). Although 5-FU was shown to have substantial antibacterial effects, the tested concentrations in these studies were much higher than what is usually present *in vivo*. Notwithstanding the fact that after an intravenous bolus injection with 5-FU, plasma concentrations in cancer patients can reach some hundreds µM, the plasma levels eventually drop to 15-30 µM after 30 minutes and to 0 µM after 2 h (Casale et al. 2004, Kosovec et al. 2008), due to the short half life time (6-22 min) of 5-FU (Bocci et al. 2000). In case of continuous infusion with 5-FU, the plasma concentrations are much lower, ranging from 3 to 10 µM and kept for a longer time period (24 h) (Joulia et al. 1999, Takimoto et al. 1999). Levels in saliva during this continuous infusion range between 0.08 and 0.8 µM (Joulia et al. 1999). It is therefore of interest to investigate the putative antimicrobial effect of physiologically relevant 5-FU concentrations on commensal microorganisms in the context of oral mucositis.

One of the main reasons for the short half life time of 5-FU is the presence of DPD in humans which breaks down 80 % of the 5-FU to dihydrofluorouracil in the liver (Diasio and Harris 1989). Patients with decreased DPD activity are more sensitive to 5-FU and are more likely to develop side effects, like mucositis, neurotoxicity and myelosuppression (Diasio et al. 1988, Harris et al. 1991, Takimoto et al. 1996). Interestingly, also some microbial species possess DPD activity (Hidese et al. 2011), which might play a role in their sensitivity to 5-FU.

For this study, several representative species of the oral cavity were selected. *S. salivarius*, *S. oralis* and *S. mitis* were included since *Streptococcus* is the most dominant genus present in the oral cavity (Dewhirst et al. 2010). Also less abundant *Lactobacillus* species *L. salivarius*, *L. oris* and *L. plantarum* were included because of their ability to produce lactic acid (Marsh and Martin 1999). *Fusobacterium nucleatum* was included as it is important in biofilm formation in the oral cavity (Zijnge et al. 2010). Furthermore, the non-pathogenic *Neisseria mucosa* was included as it can cause bacteremia in neutropenic patients developing oral mucositis (Mechergui et al. 2014). As pathogens might play an important role in mucositis, we also included *Streptococcus pyogenes*, *Pseudomonas aeruginosa* and *Klebsiella oxytoca*. *S. pyogenes* is a common oral pathogen (Wescombe et al. 2012), whereas *P. aeruginosa* and *Klebsiella* were recorded in the oral cavity of chemotherapy-treated patients (Panghal et al. 2012). Furthermore, oral mucositis has been associated with an increase in *Klebsiella* (Marsh and Martin 1999). In this study, the difference in sensitivity of different relevant oral species towards various 5-FU concentrations in the context of oral mucositis were studied. Also, the putative role of DPD in microbial resistance to 5-FU was evaluated.

2. Materials and methods

2.1 Chemicals

A filter-sterilized stock solution of 100 mM 5-FU (Sigma Aldrich, Diegem, Belgium) was prepared in dimethyl sulfoxide (DMSO) and further diluted to 50, 25, 12.5, 6.3, 3.1, 1.6, 0.8, 0.4, 0.2 and 0.1 mM. A filter-sterilized stock solution of 250 mM uracil (Sigma Aldrich, Diegem, Belgium) was prepared in DMSO and further diluted to 125, 100, 50, 32, 16, 12.8 and 6.4 mM. Stock solutions were further diluted (1:1000) in culture medium for the experiments.

2.2 Microorganisms and culture conditions

All monocultures were obtained from the Belgian Co-ordinated Collection of Micro-organisms/ LMG bacteria collection. *Streptococcus oralis* (LMG 14553), *S. salivarius* (LMG 11489), *S. mitis* (LMG 14557), *S. pyogenes* (LMG 15868) and *Klebsiella oxytoca* (LMG 3055) were cultured in Brain Heart Infusion (BHI) medium (Sigma Aldrich, Diegem, Belgium) at 37°C. *Lactobacillus salivarius* (LMG 9477), *L. oris* (LMG 9848) and *L. plantarum* (LMG 9211) were cultured in Man, Rogosa and Sharpe (MRS) medium (Oxoid, Aalst, Belgium) at 37°C. *Neisseria mucosa* (LMG 5136) was cultured in Heart Infusion medium (Sigma Aldrich, Diegem, Belgium) at 33°C. *Pseudomonas aeruginosa* (LMG 10639) in medium containing 0.45 g/L KH_2PO_4 (Carl Roth, Karlsruhe, Germany), 2.39 g/L $Na_2HPO_4.12H_2O$ (Carl Roth, Karlsruhe, Germany), 1 g/L Lab-Lemco beef extract (Oxoid, Aalst, Belgium), 2 g/L yeast extract (Oxoid, Aalst, Belgium), 5 g/L peptone (Oxoid, Aalst, Belgium) and 5 g/L NaCl (Carl Roth,

Karlsruhe, Germany) at 37°C. *Fusobacterium nucleatum* (ATCC 10953) was cultured in anaerobic Brain Heart Infusion (BHI) medium (Sigma Aldrich, Diegem, Belgium) at 37°C. Except for *Fusobacterium nucleatum*, all strains were cultured under aerobic conditions. *L. plantarum* was cultured both aerobically and anaerobically.

For the assays in anaerobic conditions, all manipulations and measurement of growth kinetics were performed in an anaerobic workstation (GP-Campus, Jacomex, TCPS NV, Rotselaar, Belgium) under a N_2:CO_2 (90:10, v/v) atmosphere. The preparation of anaerobic medium was done in Balch tubes (Glasgerätebau, Germany).

Before the start of each experiment, fresh bacteria derived from a -80°C glycerol stock were plated onto an agar plate and incubated overnight. Next, one colony of each test species was transferred into 9 mL broth and incubated in static conditions overnight at their optimal growth temperature. Subsequently, cultures were transferred (10 % v/v) into fresh broth and allowed to grow for 20 h. Cultures were then diluted to 10^4 intact cells/mL in fresh medium, as measured by flow cytometry (BD Accuri C6, Becton, Dickinson and Company, Erembodegem, Belgium) according to Van Nevel et al. (2013) before the start of the experiment.

2.3 Growth assays

Continuous growth curves were generated in 96 well plates (transparent, flat bottom) (Cell Star, Greiner Bio One, Wemmel, Belgium). In each well, a microbial suspension of 200 µL of each species (10^4 cells/mL) was treated with different concentrations of 5-FU (0.1-50 µM) and allowed to grow in static conditions at the optimal growth temperature for each species. Growth was monitored spectrophotometrically (optical density at 620 nm) by means of a Tecan Sunrise platereader (Tecan, Männedorf, Switzerland) every 30 or 60 minutes over a period of 18 to 75 h, depending on the strain. For experiments including uracil, the same experimental set-up was used, but growth was monitored by a Tecan Infinite M200 Pro (Tecan, Männedorf, Switzerland). By fitting the logistic growth model to the growth data, growth rate (µ) and maximal optical density (OD_{max}) were calculated using Grofit (version 1.1.1-1) in R (version 3.0.2) (Kahm et al. 2010). The estimation and biological errors were propagated under the assumption of normal distributed and independent errors. Each condition was performed in triplicate or quadruplicate.

2.4 Viability assays

In each well of a 96 well plate, 200 µL of a diluted bacterial suspension (10^4 cells/mL) (see previous section) was treated with different concentrations of 5-FU (0.1-50 µM) for 24 h at the optimal growth temperature of each test species. After incubation, the number of intact and damaged cells was measured by flow cytometry as described by Van Nevel et al. (2013).

For this, the samples were diluted in a filter sterile phosphate buffered solution to obtain cell numbers within the detection range (10^4-10^6 cells/mL). Next, the samples were stained with SYBR Green I (10000x diluted from stock, Invitrogen) and propidium iodide (final concentration 4 µM, Invitrogen) and incubated for 13 min at 37°C before measurement. SYBR Green penetrated all cells and resulted in a green fluorescence whereas propidium iodide penetrated only cells with a damaged cell membrane. Fluorescence resonance energy transfer (FRET) between propidium iodide and SYBR Green thus only occurred in damaged cells and generated a distinct, lower energy fluorescence signal for the damaged cells. The flow cytometer (BD Accuri C6 flow cytometer, BD, Erembodegem, Belgium) was equipped with a 488 nm solid-state laser and Milli-Q was used as sheath fluid. Signals were detected in fluorescent channels FL1 (green) and FL3 (red), respectively equipped with a 518-548 nm and 670 nm bandpass filter. Cell counts were done by measuring the number of particles in a set volume after gating on green vs. red fluorescence plots in the BD CSampler software. The gating made it possible to quantify both intact and damaged cells in each sample. We used the intact cell densities as an indicator for cell viability (Berney et al. 2007). Quality control of absolute cell counting was done with standardized beads. Background was monitored by measuring a filtered sample, equally diluted as the test samples. Each condition was performed in triplicate or quadruplicate.

2.5 Statistical analysis

Mixed-model regression of growth rate (µ), maximal OD and flow cytometer data (log cells/mL) were performed in the R (version 3.0.2) statistical environment with the concentration as categorical predictor. A random intercept effect was incorporated for each replicate measurement. In order to make correct statistical inference, all models were evaluated for normal distributed residuals with homogenous variance, by Shapiro Wilk test ($p>0.05$) and visually by Q-Q plots. Model parameters were estimated by maximum likelihood. When a significant concentration effect was present (ANOVA, $p < 0.01$), the categories were compared pair-wise by posthoc analysis using Tukey's HSD method. All tested concentrations were compared with the control condition (0 µM) and differences were considered significant at $p < 0.05$.

3. Results

3.1 Effect of 5-FU on growth of oral monocultures

To investigate the effect of 5-FU on the growth of different oral monocultures, growth curves, based on optical density, were generated (see Supplementary Figure 2.1 and Supplementary Figure 2.2) and growth rates (μ) and maximal optical densities (OD_{max}) were calculated in presence or absence of different concentrations of 5-FU (0.1-50 μM). 5-FU had no or only a minor inhibitory effect on the growth of *Lactobacillus salivarius*, *Streptococcus salivarius*, *Streptococcus mitis*, *Streptococcus oralis*, *Klebsiella oxytoca* and *Pseudomonas aeruginosa* (Figure 2.1 and Supplementary Figure 2.1). *S. salivarius* and *P. aeruginosa* did not show a response in terms of OD_{max}. For *S. salivarius,* the growth rate decreased significantly at concentrations of 5-FU higher than 6.3 μM (48-81 %). For *P. aeruginosa,* a significant decrease in growth rates could be observed at 3.1 μM 5-FU (15-58 %). *S. mitis* and *S. oralis* showed only a significant decrease in OD_{max} (67 and 64 % respectively) at the highest tested concentration (50 μM 5-FU) and a decrease in growth rate starting from 6.3 μM (38-75 %) and 12.5 μM (32-64 %), respectively. *K. oxytoca* showed a significant decrease in OD_{max} (30-35 %) for 12.5-50 μM; for growth rates only a significant decrease was seen at 6.3 μM (37 %) but not at higher concentrations. For *L. salivarius* there was distinct variability in the data, but a significant decrease in growth rate was seen at 6.3 μM 5-FU (75 %) and a decrease of 53-57 % (not significant) was seen at 12.5-25 μM 5-FU.

Lactobacillus oris, *Lactobacillus plantarum* (aerobic/anaerobic), *Streptococcus pyogenes*, *Fusobacterium nucleatum* and *Neisseria mucosa* appeared to be more sensitive to 5-FU (Figure 2.2 and Supplementary Figure 2.2). For *L. oris* a significant decrease in OD_{max} (22-32 %) was seen for concentrations ranging from 0.8 to 12.5 μM 5-FU, whereas at 50 μM no growth was detected. For *L. plantarum* (aerobic) only a significant decrease in growth rate was seen at concentrations higher than 3.1 μM (57-70 %), although decreases of 19-41 % (non-significant) were seen at 0.4 - 1.6 μM. *L. plantarum* (anaerobic) showed significant decrease for all tested concentrations (0.1 - 25 μM) for both growth rate as well as OD_{max}. No growth was detected for concentrations starting from 6.3 μM. *S. pyogenes* did not show a response in terms of OD_{max}, but a significant decrease in growth rate at concentrations higher than 0.4 μM 5-FU (33-72 %) was observed. For *F. nucleatum* a significant decrease in OD_{max} and growth rate was seen for 0.8 and 0.4 μM respectively and no growth was detected for concentrations higher than 1.6 μM 5-FU. For *N. mucosa* significant decreases in OD_{max} and growth rate were detected for concentrations higher than 0.8 and 0.2 μM respectively and no growth was detected above 12.5 μM 5-FU.

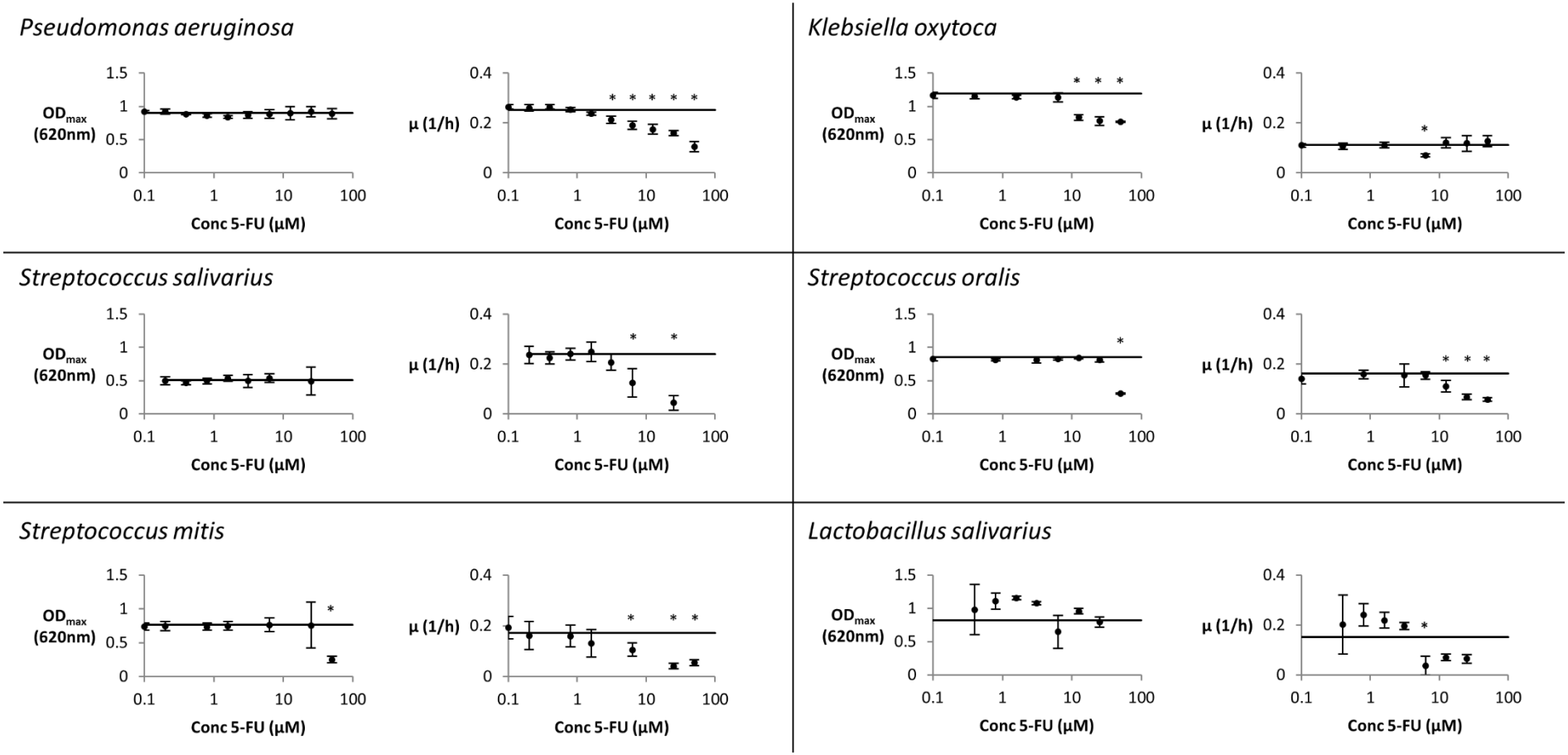


Figure 2.1 - 5-FU has only a minor negative effect on growth of *K.* oxytoca, *S. salivarius*, *S. mitis*, *S. oralis*, *P. aeruginosa* and *L. salivarius*.

Maximal optical density (OD_{max}) and growth rate (μ), calculated from growth curves of oral monocultures treated with different concentrations of 5-FU (0.1-50 μM) (AV ± SD); —— = control condition (0 μM)). Significant deviations from the control condition (0 μM) are indicated by the asterisks ($p<0.05$).

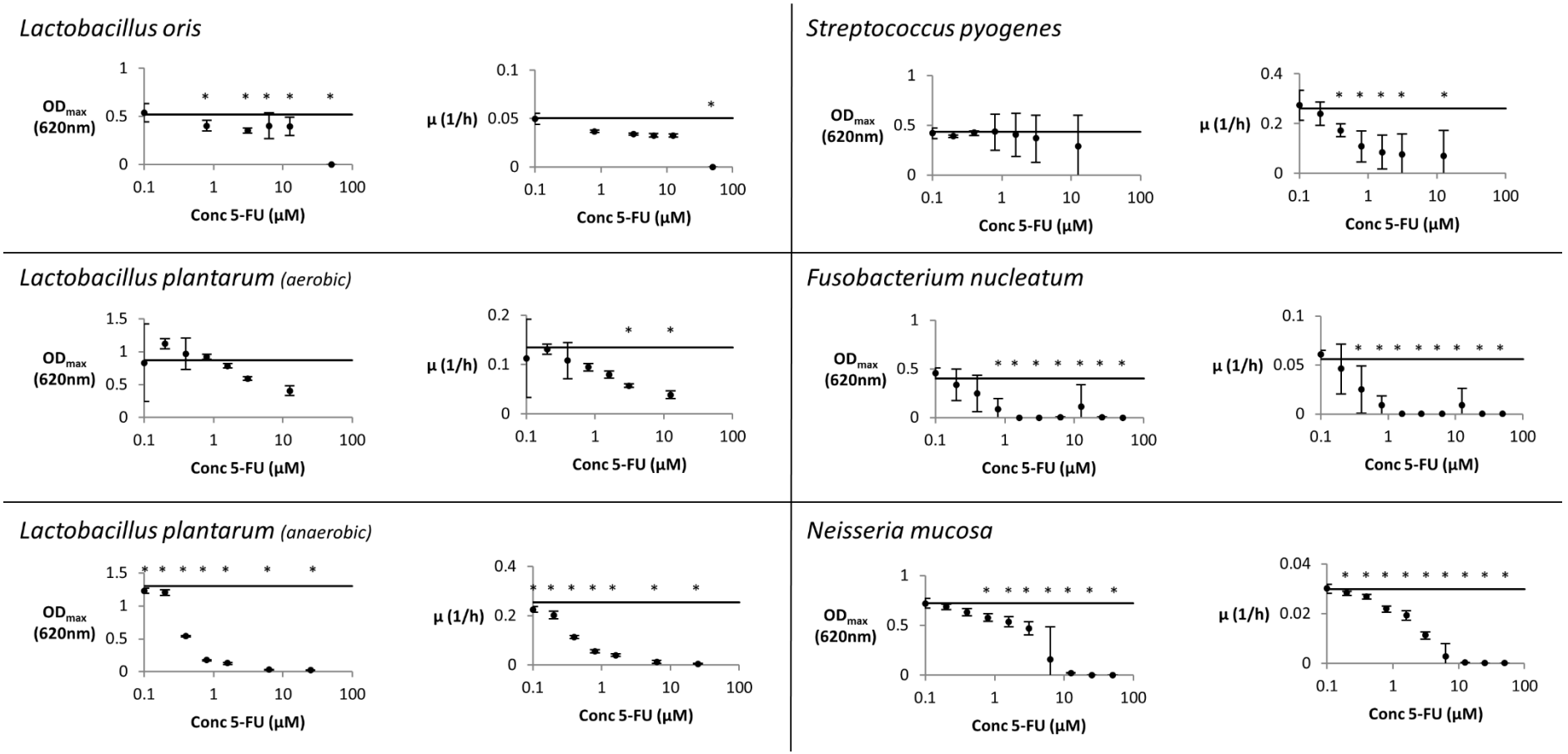


Figure 2.2 - 5-FU has a major negative effect on the growth of *L. oris*, *L. plantarum* (aerobic/anaerobic), *S. pyogenes*, *F. nucleatum* and *N. mucosa.*
Maximal optical density (OD_{max}) and growth rate (μ), calculated from growth curves of oral monocultures treated with different concentrations of 5-FU (0.1-50 μM) (AV ± SD; —— = control condition (0 μM)). Significant deviations from the control condition (0 μM) are indicated by the asterisk ($p<0.05$).

3.2 Effect of 5-FU on viability of oral monocultures

After 24 h of treatment, the monocultures were stained with SYBR Green/propidium iodide to evaluate the effect of different physiological concentrations of 5-FU on cell viability. This staining made it possible to quantify both intact and damaged cells in each sample. We used the intact cell densities as an indicator for cell viability (Berney et al. 2007).

Since the number of damaged cells of all tested monocultures (except for *L. salivarius*) was lower than the background, these data were not presented in the graphs. Some species showed only a minor decrease in the number of viable cells (Figure 2.3). For *K. oxytoca*, *S. salivarius*, *S. oralis* and *P. aeruginosa* the amount of viable cells decreased with less than 0.65, 1.19, 1.03, 0.65 log units respectively at a concentration range between 12.5-50 µM. Despite the high variability in the counts of *S. mitis* cells, numbers generally did not drop more than 2.4 log units or below the background value. The number of intact cells for *L. salivarius* decreased with 0.8 log units at 25 µM, coinciding with an increase of damaged cells with 1.6 log units starting from 3.1 µM.

The most sensitive test species appeared to be *F. nucleatum*, *L. oris*, *L. plantarum*, *N. mucosa* and *S. pyogenes* (Figure 2.4). For *L.* oris and *N. mucosa* there was a decrease in viable cells above 1.6 µM and going below the background at 25 and 6.3 µM respectively. *L. plantarum* (aerobic/anaerobic), *S. pyogenes*, and *F. nucleatum* were even more sensitive and showed a decrease for concentrations higher than 0.2, 0.4, 0.4 and 0.4 µM 5-FU respectively and going below the background at 0.4, 0.8, 0.4 and 0.8 µM respectively.

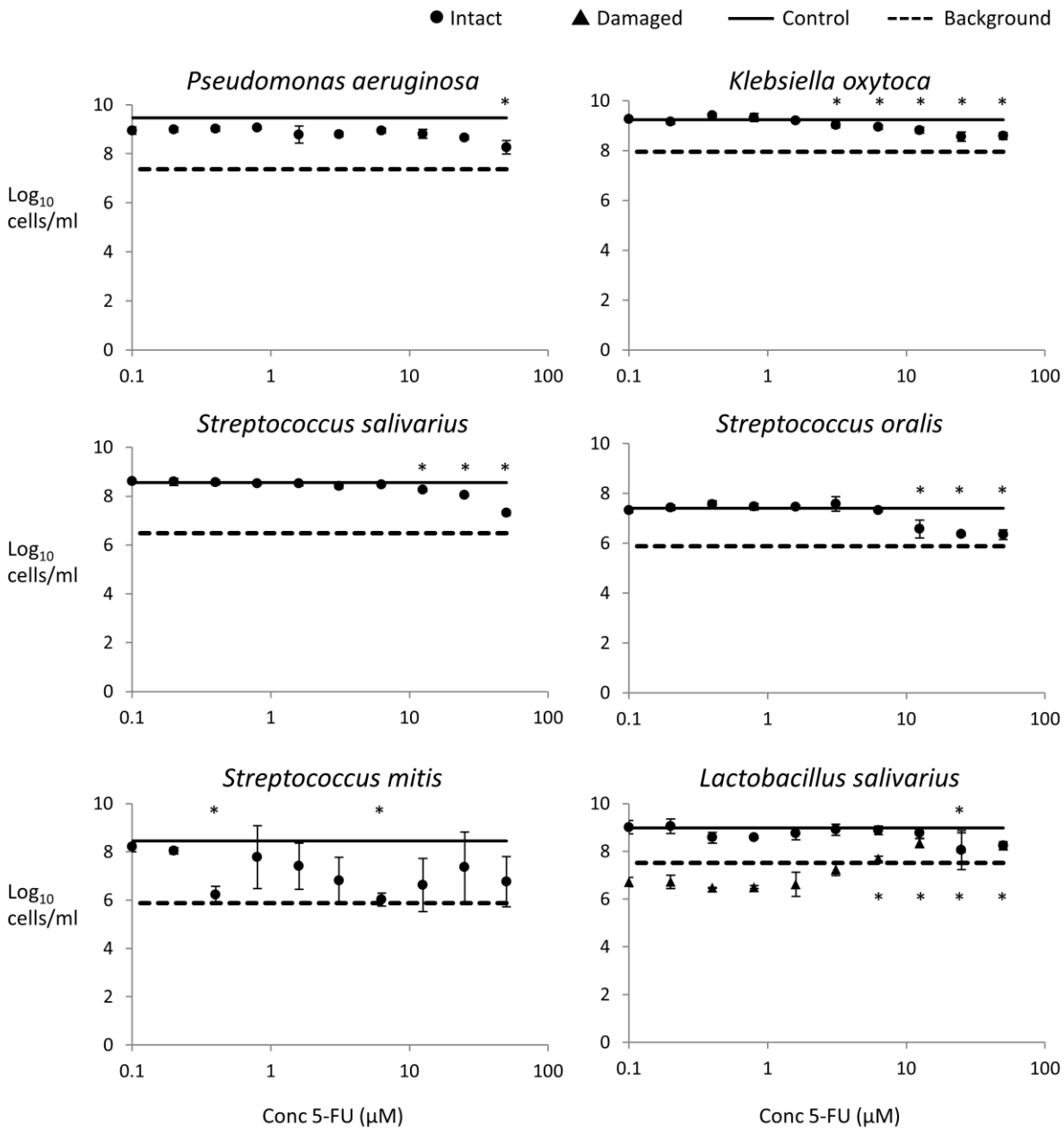


Figure 2.3 - 5-FU has a minor effect on viable cell counts above 25 µM for *K. oxytoca*, *S. salivarius*, *S. oralis*, *S. mitis*, *P. aeruginosa*, whereas *L. salivarius* had more damaged cells after treatment with 3.1-50 µM 5-FU. Flow cytometric analysis of viability of oral monocultures treated with different concentrations of 5-FU (AV ± SD). Significant deviations from the control condition (0 µM) are indicated by the asterisks ($p<0.05$). The background (dotted line) represents a filtered sample.

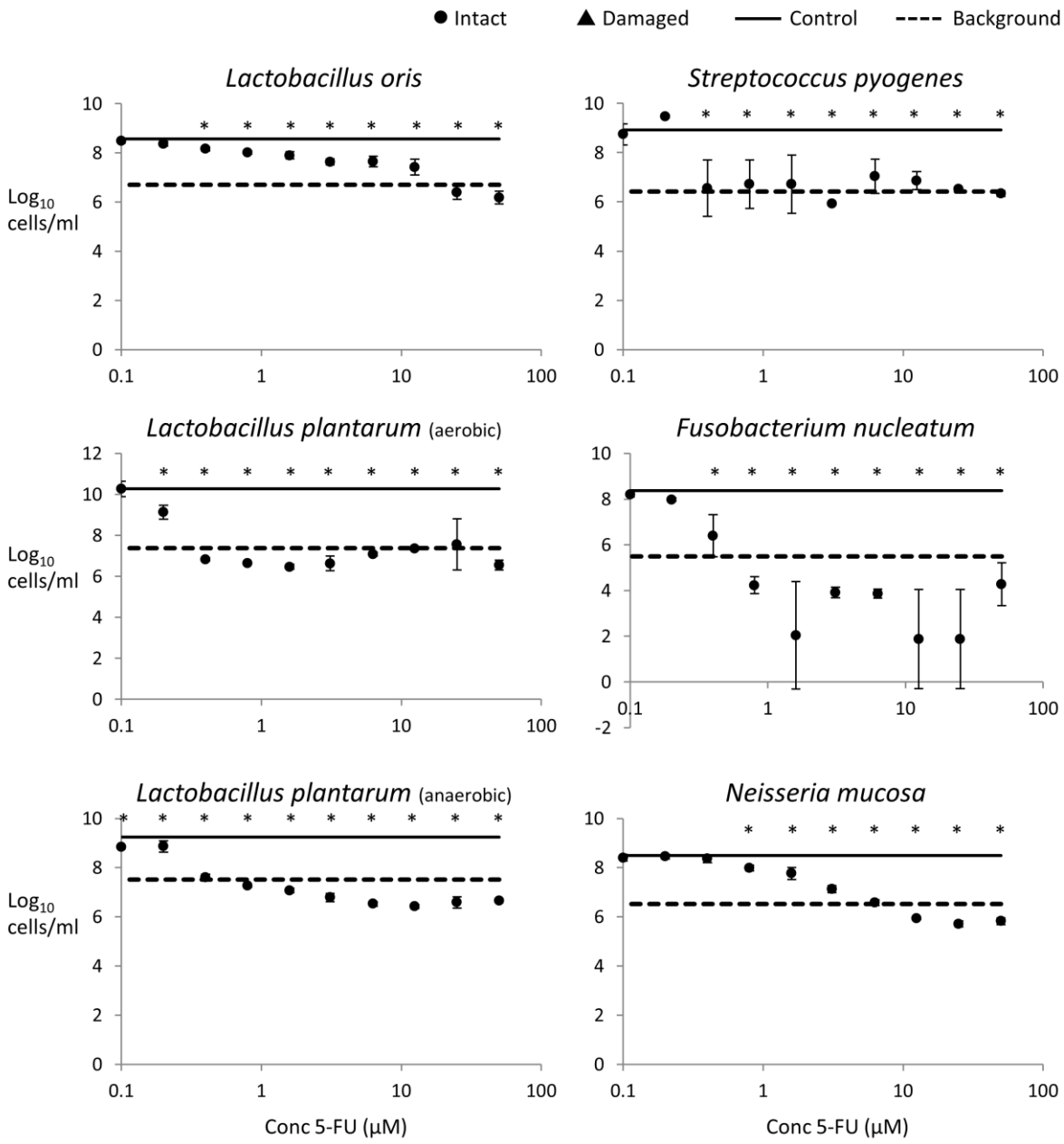


Figure 2.4 - 5-FU causes a major decrease in viable cell counts starting from 0.4-1.6 µM for *L. oris*, *L. plantarum* (aerobic/anaerobic), *S. pyogenes*, *F. nucleatum* and *N. mucosa.*

Flow cytometric analysis of viability of oral monocultures treated with different concentrations of 5-FU (AV ± SD). Significant deviations from the control condition (0 µM) are indicated by the asterisks ($p<0.05$). The background (dotted line) represents a filtered sample.

3.3 Effect of 5-FU on growth of oral monocultures in presence of uracil

To investigate the role of DPD in the sensitivity response towards 5-FU, growth experiments were performed with two resistant species, namely *Streptococcus salivarius* and *Pseudomonas aeruginosa* in the presence and absence of uracil, an inhibitor of DPD. DPD is normally involved in the degradation of 5-FU into the non-toxic dihydrofluorouracil. Quantification of 5--FU and dihydrofluorouracil was not possible by HPLC analysis, due to high background (data not shown). Hence, we hypothesize that 5-FU resistant microorganisms might become more sensitive to 5-FU in the presence of uracil, a competitive substrate of DPD, provided that the activity of 5-FU is DPD-mediated. Therefore, growth curves were generated for 0 µM, 1.6 µM and 12.5 µM of 5-FU with or without uracil (5-FU:uracil ratio of 1:4) (similar results were obtained with a 1:10 ratio; data not shown). At 1.6 µM 5-FU, uracil did not modulate the resistance of both test species towards 5-FU (data not shown). Remarkably, at 12.5 µM, uracil stimulated growth in the presence of 5-FU rejecting our hypothesis that DPD was involved in the microbial resistance towards 5-FU (Figure 2.5; p-values see Supplementary Table 2.1 and Supplementary Table 2.2).

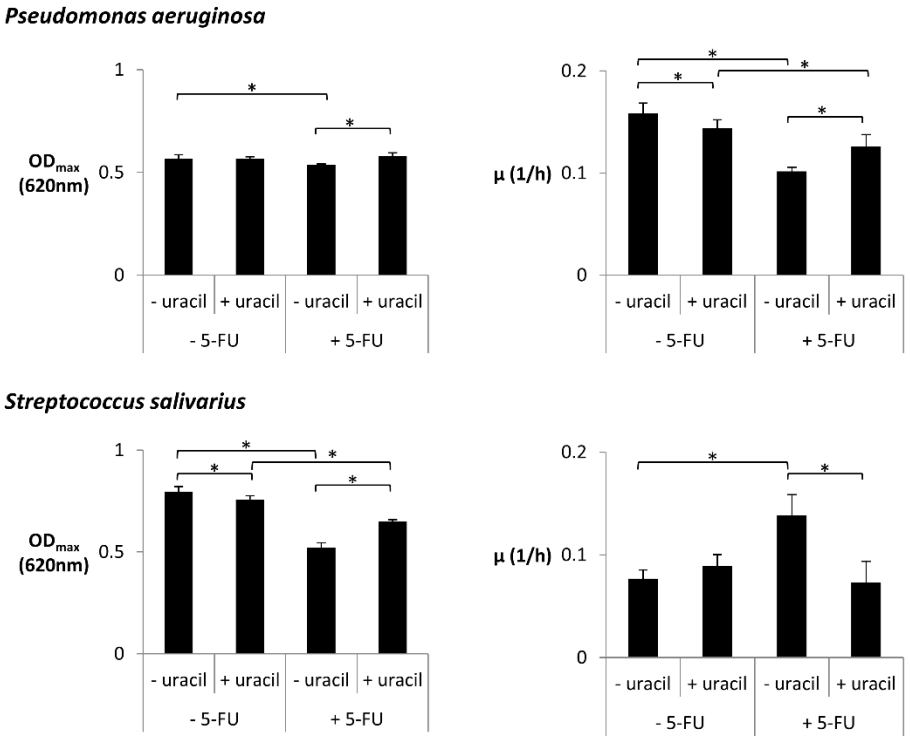


Figure 2.5 - At 12.5 µM, 5-FU had a small negative effect on the growth of *P. aeruginosa* and *S. salivarius* and by adding uracil, they both became a bit more resistant to 5-FU.
Maximal optical density (OD_{max}) and growth rate (µ) calculated from growth curves of *P. aeruginosa* and *S. salivarius* treated with or without 5-FU (12.5 µM) and with or without uracil (50 µM) (AV ± SD). Relevant significant deviations are indicated by the asterisks ($p<0.05$).

4. Discussion

5-FU is one of the oldest chemotherapeutic agents and causes multiple side effects such as oral mucositis. It is known to have an antibacterial effect at high concentrations, but its effect at physiologically relevant concentrations is still underexplored. In this study, we show that at low concentrations (0.1-50 µM) there is a great variability in 5-FU sensitivity among oral microorganisms. The combination of growth and viability test results suggests that oral microorganisms can be divided in two sensitivity groups: the resistant microorganisms and the sensitive microorganisms. The first group consists of *Klebsiella* oxytoca, *Streptococcus salivarius*, *Streptococcus mitis*, *Streptococcus oralis* and *Pseudomonas aeruginosa* which only experience a minor decrease in growth and viability following exposure to high concentrations of 5-FU (12.5-50 µM). The second group comprises *Lactobacillus* oris, *Lactobacillus plantarum* (aerobic/anaerobic), *Lactobacillus salivarius*, *Streptococcus pyogenes*, *Neisseria mucosa* and *Fusobacterium nucleatum* which appear to be more sensitive to 5-FU with clearly negative effects on both growth and viability starting from 0.4-3.1 µM . One of the sensitive species is somewhat particular, namely *L. salivarius* for which there is a distinct increase in damaged cells at higher concentrations of 5-FU, measured by flow cytometry.

Our finding of differential microbial sensitivity to 5-FU correlates with previous *in vivo* observations. In a rat mucositis study, Stringer et al. (2009c) identified *Pseudomonas aeruginosa* and *Escherichia coli* as 5-FU resistant species. Further, *P. aeruginosa* and *E. coli* were shown to be able to overgrow the oral community and penetrate into the damaged underlying mucosa thereby causing local infections (Stringer et al. 2009c). Another study with rats reported similar shifts, with Gram-negative rods becoming more abundant upon treatment with 5-FU (von Bultzingslowen et al. 2003). Also other chemotherapeutics have been shown to generate differential microbial response leading to infections as demonstrated for pathogenic *Pseudomonas aeruginosa* in leukemic patients (Goldschmidt and Bodey 1972). Therefore, microbial 5-FU resistance should be considered as an important risk factor for infections especially in the context of oral mucositis.

Previous studies investigating the antibacterial properties of 5-FU primarily evaluated concentrations that are not representative of *in vivo* 5-FU concentrations during chemotherapy. Plasma concentrations after continuous infusion range from 0.1 to 8.8 µM, depending on the dose (300 – 2300 mg/m²/day) (Grem 2000). In the mouth, saliva levels during continuous infusion range from 0.08 to 0.8 µM (Joulia et al. 1999), hence significantly lower than what can be measured in plasma but still high enough to affect the most sensitive microorganisms in the mouth. Furthermore, plasma concentrations can reach much higher concentrations in DPD deficient patients due to the 10 times longer half life time of 5-FU (Saif et al. 2009).

In humans, DPD seems to play an important role in obtaining resistance to 5-FU since it can break down 5-FU to the non-toxic dihydrofluorouracil (Aziz et al. 2013). Literature data on microbial DPD is scarce, but the presence of DPD activity has previously been shown for *Pseudomonas aeruginosa* (Kim and West 1991) and *Escherichia coli* (West 1998). In our study, *K. oxytoca*, *S. salivarius*, *S. mitis*, *S. oralis* and *P. aeruginosa* were shown to be 5-FU resistant. However, a search of the NCBI protein database on all tested microorganisms, indicated that theoretically only *P. aeruginosa* and *L. oris* contain a DPD enzyme. Hence, no clear link between microbial DPD and 5-FU sensitivity could be identified. In clinical settings a combination of 5-FU and uracil, an inhibitor of DPD, is used to improve the efficiency of 5-FU. Uracil competitively binds to DPD, reducing the degradation of 5-FU to dihydrofluorouracil (Takechi et al. 1997, Omura 2003). In our study, we applied the same principle to test if microbial 5-FU sensitivity is dependent on the presence of DPD in resistant microorganisms, Yet our results suggest that DPD is not a key-enzyme.

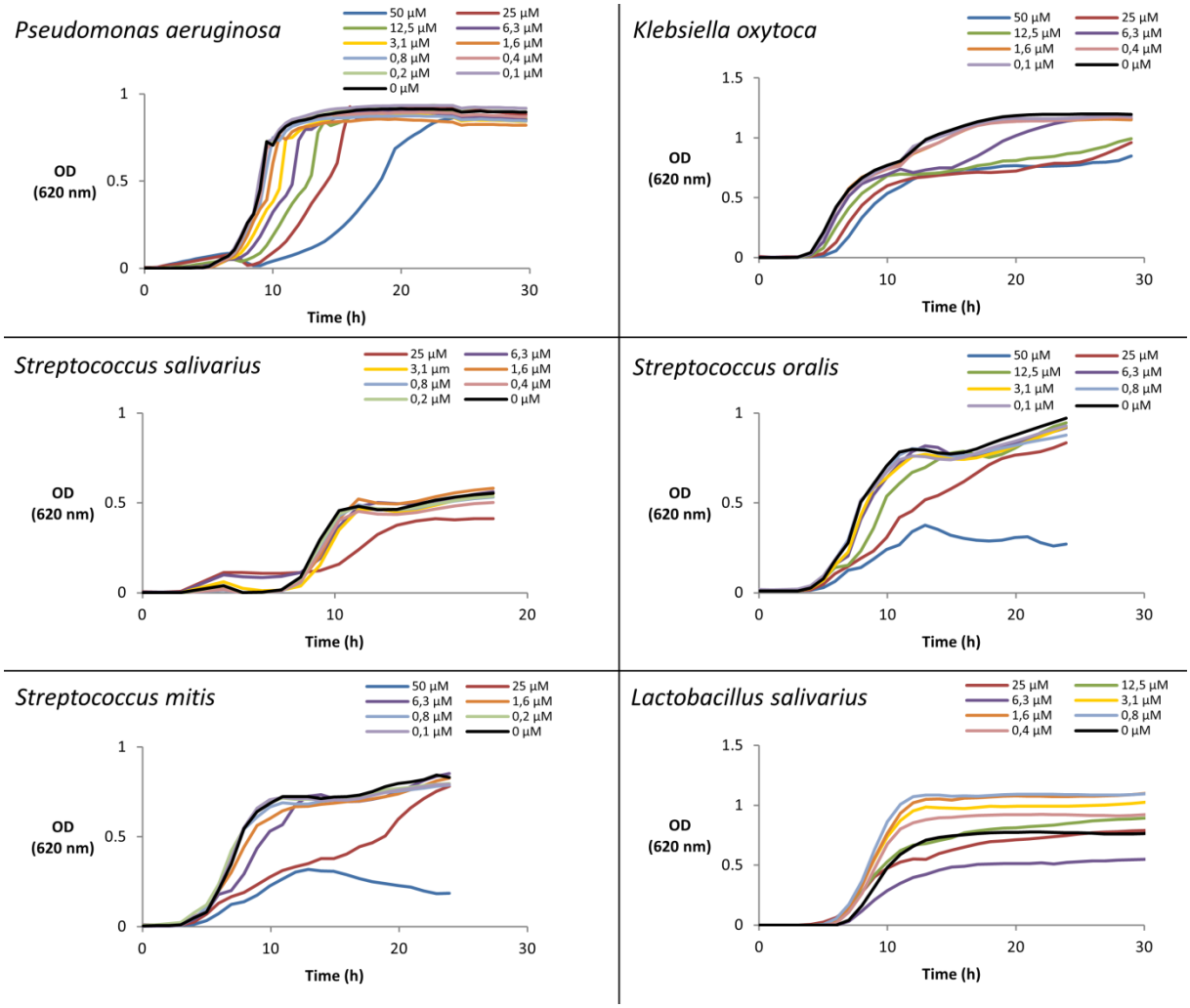
Many other enzymes have been reported to be involved in the pharmacokinetics of 5-FU, including thymidylate synthase (TS). TS provides the sole *de novo* production of thymidylate and is inhibited by 5-FU (Longley et al. 2003). In cell lines a positive relationship exists between the DPD and TS levels and 5-FU sensitivity (Beck et al. 1994). In *C. elegans*, overexpression of TS and DPD leads to a higher survival after treatment with 5-FU (Kim et al. 2008). Also in humans, overproduction of TS results in 5-FU resistance (Clark et al. 1987, Johnston et al. 1995). Interestingly, a search for TS presence in the NCBI protein database in our study showed hits for all tested species. Therefore, TS and other enzymes might play a role in 5-FU resistance. At least for the treatment of colorectal cancer patients, the analysis of three predictive markers (TS, DPD and thymidine phosphorylase (TP)) is used to predict 5-FU efficacy (Salonga et al. 2000).

In conclusion, our study shows that 5-FU sensitivity varies among different oral microorganisms and that 2 clear groups can be distinguished: resistant and sensitive microorganisms. Some species such as *L.* oris, *L. plantarum*, *L. salivarius*, *S. pyogenes*, *N. mucosa* and *F. nucleatum* are sensitive at concentrations as low as 0.4 µM, which can be measured in blood and saliva during continuous 5-FU treatment. We also provided evidence that the DPD enzyme is probably not responsible for microbial resistance to 5-FU. Our data further indicate that oral mucositis patients are likely to develop infections caused by an overgrowth of 5-FU resistant strains such as the multi-drug resistant *P. aeruginosa* and dysbiosis of the mucosa. To get a complete picture of the impact of 5-FU on the oral microbiome, an ecosystemic approach is needed in further research.

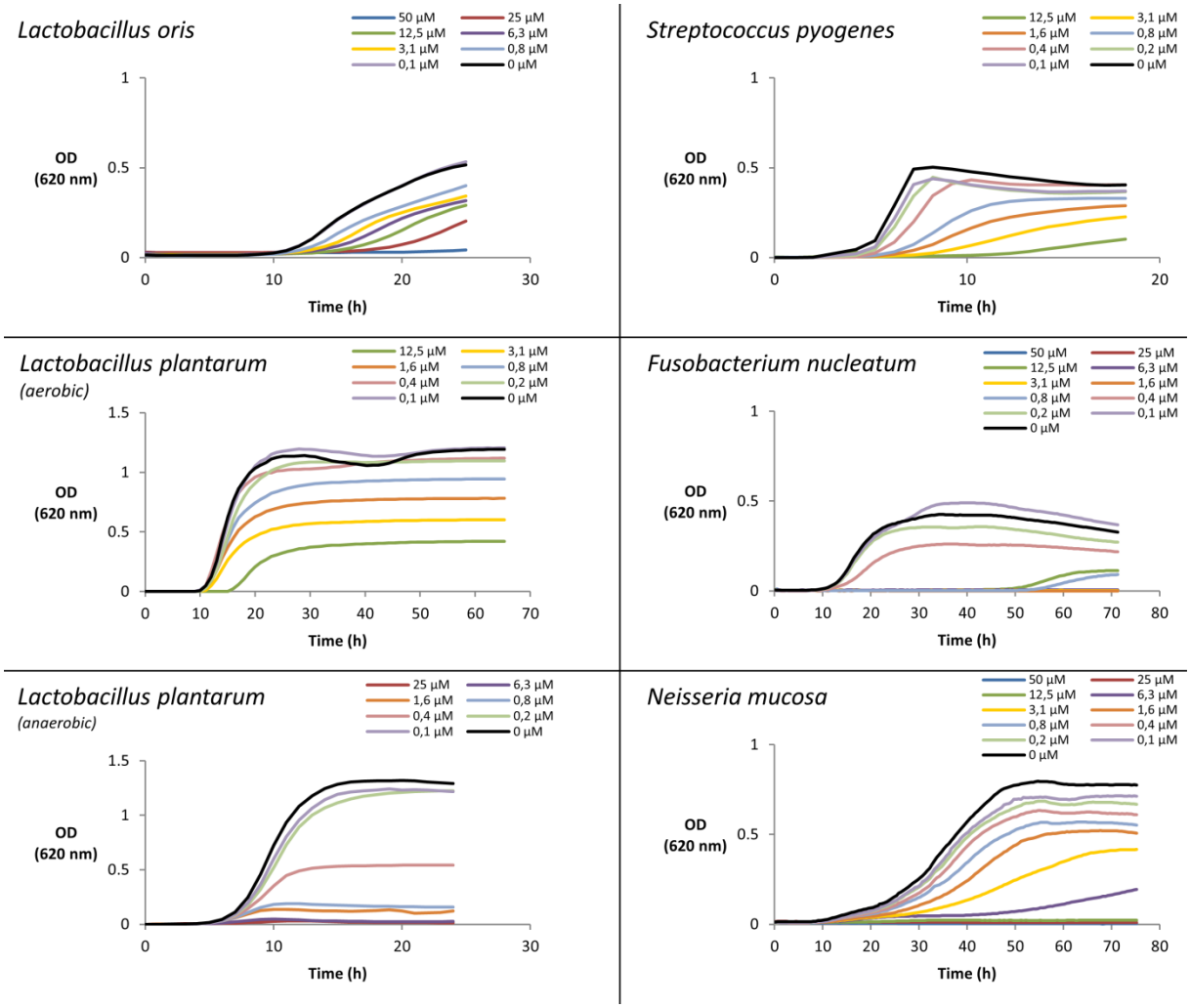
5. Acknowledgements

E.V. is a doctoral research fellow supported by the Bijzonder Onderzoeksfonds (Ghent University; B/13807/01–BOF13/DOC/280). B.V. was funded by the Seventh Framework Programme (FP7/2011) under grant agreement n° 299169 (Mucositis Platform). The authors would like to thank Jan Arends and Kim De Paepe for their review of the manuscript.

6. Supplementary information



Supplementary Figure 2.1 - Growth curves of *Klebsiella oxytoca*, *Streptococcus salivarius*, *Streptococcus mitis*, *Streptococcus oralis*, *Pseudomonas aeruginosa* and *Lactobacillus salivarius* treated with different concentrations of 5-FU.



Supplementary Figure 2.2 - Growth curves of *Lactobacillus oris*, *Lactobacillus plantarum* (aerobic/anaerobic), *Streptococcus pyogenes*, *Fusobacterium nucleatum* and *Neissera mucosa* treated with different concentrations of 5-FU.

Supplementary Table 2.1 - P-values of maximal optical density (OD_{max}) and growth rate (µ) calculated from growth curves of *P. aeruginosa* treated with or without 5-FU (12.5 µM) and with or without uracil (50 µM).

P. aeruginosa		- 5-FU		+ 5-FU	
OD_{max}	µ	-uracil	+ uracil	-uracil	+ uracil
- 5-FU	- Uracil	/	**<1e-04**	**<1e-04**	**<1e-04**
	+ uracil	1	/	**<1e-04**	**<1e-04**
+ 5-FU	- Uracil	**<0,001**	**<0,001**	/	**<1e-04**
	+ uracil	0,406	0,385	**<0,001**	/

Supplementary Table 2.2 - P-values of maximal optical density (OD_{max}) and growth rate (µ) calculated from growth curves of *S. salivarius* treated with or without 5-FU (12.5 µM) and with or without uracil (50 µM).

S. salivarius		- 5-FU		+ 5-FU	
OD_{max}	µ	-uracil	+ uracil	-uracil	+ uracil
- 5-FU	- Uracil	/	0,460	**<1e-4**	0,974
	+ uracil	**0,001**	/	**<1e-4**	0,233
+ 5-FU	- Uracil	**<1e-4**	**<1e-4**	/	**<1e-4**
	+ uracil	**<1e-4**	**<1e-4**	**<1e-4**	/

.

CHAPTER 3

5-Fluorouracil and irinotecan (SN-38) have limited impact on colon microbial functionality and composition *in vitro*

This chapter has been redrafted after

Vanlancker, E., Vanhoecke, B., Stringer, A. and Van de Wiele, T. 5-Fluorouracil and irinotecan (SN-38) have limited impact on colon microbial functionality and composition *in vitro*. *Submitted at PeerJ.*

CHAPTER 3

5-Fluorouracil and irinotecan (SN-38) have limited impact on colon microbial functionality and composition *in vitro*

Abstract

Gastrointestinal mucositis is a debilitating side effect of chemotherapy, with currently no treatment available. As changes in microbial composition have been reported upon chemotherapeutic treatment *in vivo*, it is thought that gut microbiota contribute to the mucositis etiology. Yet, it is not known whether chemotherapeutics directly cause microbial dysbiosis, thereby increasing mucositis risk, or whether the chemotherapeutic subjected host environment disturbs the microbiome thereby aggravating the disease. To address this question, we used the M-SHIME®, an *in vitro* mucosal simulator of the human intestinal microbial ecosystem, as an experimental setup that excludes the host factor. The direct impact of two chemotherapeutics, 5-fluorouracil (5-FU) and irinotecan, on the luminal and mucosal gut microbiota from several human donors were investigated through monitoring fermentation activity and next generation sequencing. At a dose of 10 µM in the mucosal environment, 5-FU impacted the functionality and composition of the colon microbiota to a minor extent. Similarly, a daily dose of 10 µM SN-38 in the luminal environment did not cause significant changes in the functionality or microbiome composition. As our mucosal model does not include a host compartment, we conclude that a putative microbial contribution to mucositis is initially triggered by an altered host environment upon chemotherapy.

1. Introduction

5-Fluorouracil (5-FU) and irinotecan (SN-38) are two commonly used chemotherapeutic agents for cancer treatment. A major side effect of these agents is gastrointestinal mucositis, an inflammation and ulceration of the gastrointestinal mucosa and mostly diagnosed based on the occurrence of diarrhea (Benson et al. 2004, Peterson et al. 2015). The incidence of chemotherapy-induced diarrhea associated with 5-FU or irinotecan treatment varies around 50-80 % (Benson et al. 2004). Treatment of colon cancer with FOLFOX (5-FU, leucovorin, and oxaliplatin) or FOLFIRI (5-FU, leucovorin and irinotecan) or IROX (Irinotecan and oxaliplatin) results in a risk for grade 3 or 4 diarrhea (severe diarrhea requiring hospitalization or having life-threatening consequences, according to the National Cancer Institute Common Terminology Criteria of Adverse Events (NCI CTCAE) classification) of 10 %, 10 % and 24 %, respectively (Keefe et al. 2007). While gastrointestinal mucositis often causes cessation of the cancer treatment and a lot of discomfort for the patient, no effective treatment is available yet. The MASCC/ISOO has put an effort in formulating guidelines and recommendations on how to prevent and treat gastrointestinal mucositis, such as by the use of octreotide for the treatment of diarrhea associated with HSCT (Lalla et al. 2014).

In research on the pathobiology of mucositis more and more interest is going to the role and/or the effect of the gut microbiota during chemotherapeutic treatment (van Vliet et al. 2010, Stringer 2013). Microbiota have an important function in many pathways some of which are also involved in the development of gastrointestinal mucositis. For example, microbiota influence intestinal permeability and thickness of the mucus layer, both important in barrier function during mucositis (van Vliet et al. 2010, Touchefeu et al. 2014). Next, recognition of microbial antigens to TLR can activate the NF-κB pathway, resulting in production of pro-inflammatory cytokines that are crucial in the mucositis pathobiology (van Vliet et al. 2010, Touchefeu et al. 2014, Vanhoecke and Stringer 2015). Under healthy conditions commensal microorganisms are thought to live in a homeostatic relationship with the host providing a low grade immune activation and stimulating epithelial repair in an NF-κB dependent pathway (van Vliet et al. 2010, Touchefeu et al. 2014). But during cancer treatment, changes at the level of the host and/or microbiome may disturb this homeostatic relationship and increase the inflammatory status.

Animal and human studies have repeatedly shown that chemotherapeutics can change the gut microbiota (Stringer 2013, Touchefeu et al. 2014). In rat models, both 5-FU (von Bultzingslowen et al. 2003, Stringer et al. 2009c) and irinotecan (Stringer et al. 2007, Stringer et al. 2008, Stringer et al. 2009a, Lin et al. 2012) modified the gut microbiome, with a decrease in commensal microbiota and increases in *Escherichia* spp., *Clostridium* spp. and *Enterococcus* spp. Clinical studies report on shifts of fecal microbiota upon chemotherapeutic

treatment (Touchefeu et al. 2014), with a lower diversity and abundance of microbiota observed after chemotherapy. Most frequent changes are decreases in *Bifidobacterium*, *Faecalibacterium* and *Clostridium* cluster XIVa and increases in *Bacteroides* and *Escherichia* (van Vliet et al. 2009, Zwielehner et al. 2011, Stringer et al. 2013, Montassier et al. 2014). Yet, besides impacting the microbiota, chemotherapeutics also affect the mucus layer and the number of goblet cells which is likely the result of an increase in pro-inflammatory cytokine levels and leading to an altered mucosal barrier (Stringer 2013).

To further unravel the role of microbiota in gastrointestinal mucositis, it is important to understand the direct impact of chemotherapeutics on the functionality and composition of the gut microbiome. While animal and human studies do not allow to distinguish between host and microbiome effects, the goal of the present study was to investigate the direct effect of two chemotherapeutics, 5-FU and SN-38, on the gut microbiome. Therefore we used an *in vitro* model that was proven to be representative for the human colon microbiome: the M-SHIME® model (Mucosal-Simulator of the Human Intestinal Microbial Ecosystem) (Van den Abbeele et al. 2012).

2. Materials and methods

2.1 Chemicals

A filter-sterilized stock solution of 10 mM 5-FU (Sigma Aldrich, Diegem, Belgium) was prepared in DMSO. The stock solution was further diluted (1:1000) in the mucus solution to a final concentration of 10 µM.

A filter-sterilized stock solution of 10 mM SN-38 (Sigma Aldrich, Diegem, Belgium) was prepared in DMSO. The stock solution was further diluted (1:1000) in the medium to a final concentration of 10 µM.

2.2 M-SHIME

2.2.1 Experimental set-up

The M-SHIME® (Mucosal-Simulator of the Human Intestinal Microbial Ecosystem, joint registered name from Ghent University and ProDigest) is an *in vitro* dynamic model for the human intestinal tract, incorporating both luminal and mucosal colon environment, resulting in distinct luminal and mucosal microbial populations (Van den Abbeele et al. 2012). The set-up used in this study consisted of a stomach/small intestine vessel and two proximal colon vessels (control and treatment) for six human donors in parallel (Figure 3.1).

Fecal samples were collected and prepared within 1 h according to standard procedures (Molly et al. 1993). In short, aliquots (20 g) of fresh fecal samples were diluted and

homogenized with 100 mL 0.1 M phosphate buffer (8.8 g/L K_2HPO_4. and 6.8 g/L KH_2PO_4., pH 6.8) containing 1 g/L sodium thioglycolate as reducing agent. After removal of the particulate material by centrifugation (2 min, 500 g), each colon vessel was inoculated with 40 mL of the fecal suspension.

The double-jacketed vessels were kept at 37°C and flushed daily with N_2 (5 min) to assure anaerobic conditions. All colon compartments (500 mL) were stirred (200 rpm) and pH-controlled (pH 5.6-5.9). Mucosal conditions were created as described by Van den Abbeele et al. (2012). Briefly, 80 mucin-agar covered microcosms (AnoxKaldnes K1 carrier; AnoxKaldnes AB, Lund, Sweden) were brought in a polyethylene netting (Zakkencentrale, Rotterdam, the Netherlands) (Van den Abbeele et al. 2012). Half of the microcosms were replaced every other day with fresh sterile ones under a flow of N_2 to maintain anaerobic conditions. After washing the beads twice with phosphate buffered solution (PBS) to discard luminal microbiota, samples of the mucin-agar were taken and stored at -20°C.

After an initial incubation of 16 h, pumps were switched on in order to supply each colon compartment with 140 mL nutritional medium and 60 mL pancreatic juice three times a day. The nutritional medium contained (in g/L) yeast extract (3.0), special peptone (1.0), mucin (2.0), arabinogalactan (0.25), pectin from apple (0.5), xylan (0.25), potato starch (1.0). The pancreatic juice was prepared as described earlier by Van den Abbeele et al. (2012). The treatment started after two days of stabilization.

Microbial fermentation activity of the proximal colon (SCFA production) and community composition (ratio Firmicutes/Bacteroidetes/Proteobacteria, Supplementary Table 3.1 and Supplementary Table 3.2) was shown to be consistent with that of previous SHIME runs (Molly et al. 1994, Geirnaert et al. 2015).

2.2.2 Experimental design M-SHIME with 5-FU

In this set-up we compared a challenge of the *in vitro* cultured human microbiota with 5-FU to a control situation (Figure 3.1A). As 5-FU is intravenously supplemented to the patient and reaches the gut through the mucus layer, we chose to dose 5-FU directly in the mucus-covered microcosms. Taking into account pharmacokinetics during continuous infusion with 5-FU, a representative serum concentration range of 5-FU *in vivo* is 3-10 µM. We chose the highest concentration to evaluate the effect to mucosal 5-FU towards the gut microbiome. DMSO was used as a negative control. On day 0, 2 and 4, half of the mucin-covered microcosms were replaced with new treated ones. Therefore, three doses of 5-FU were given in total. To cover biological reproducibility, we compared six different donors, all healthy volunteers who had no history of antibiotic treatment up to 6 months prior to the study (Ethical approval from Ghent University hospital, Belgian Registration number BE 6700201214538).

Luminal samples were taken every 24 h and mucosal samples every 48 h (on day 2, 4 and 6). For donor 1 and 2, additional luminal samples were taken 6 h after each treatment.

2.2.3 Experimental design M-SHIME with SN-38

In this set-up we compared a challenge of the *in vitro* cultured human microbiota with SN-38 to a control situation (Figure 3.1B). As SN-38 enters the small intestine via the bile, the treatment was added daily to the luminal environment, just before the feed entered the colon compartments. As an average colon concentration was estimated on 1- 2 µM, based of fecal concentrations of SN-38, a concentration of 10 µM was used to make sure testing the highest physiological relevant concentration. The M-SHIME system was treated with SN-38 for 6 consecutive days. DMSO without SN-38 was used as a negative control. On day 0, 2 and 4, half of the mucin-covered microcosms were replaced with new ones (non-treated). To cover biological reproducibility, we compared five different donors (healthy volunteers) who had no history of antibiotic treatment up to 6 months prior to the study (Ethical approval from Ghent University hospital, Belgian Registration number BE 6700201214538). Both luminal and mucosal samples were taken every 48 h.

2.3 Short chain fatty acids (SCFA)

Luminal samples were diluted 1:2 to a total volume of 2 mL and the SCFA were extracted with diethyl ether and analyzed using a gas chromatograph as described by De Weirdt et al. (2010). The concentration of acetate, propionate, butyrate, isobutyrate, valerate, isovalerate, caproate and isocaproate was determined in each sample and the total amount of SCFA was calculated as the sum of all. The relative concentration of each SCFA was expressed as mol % being the ratio of its concentration (mM) and the total SCFA concentration (mM) multiplied by 100.

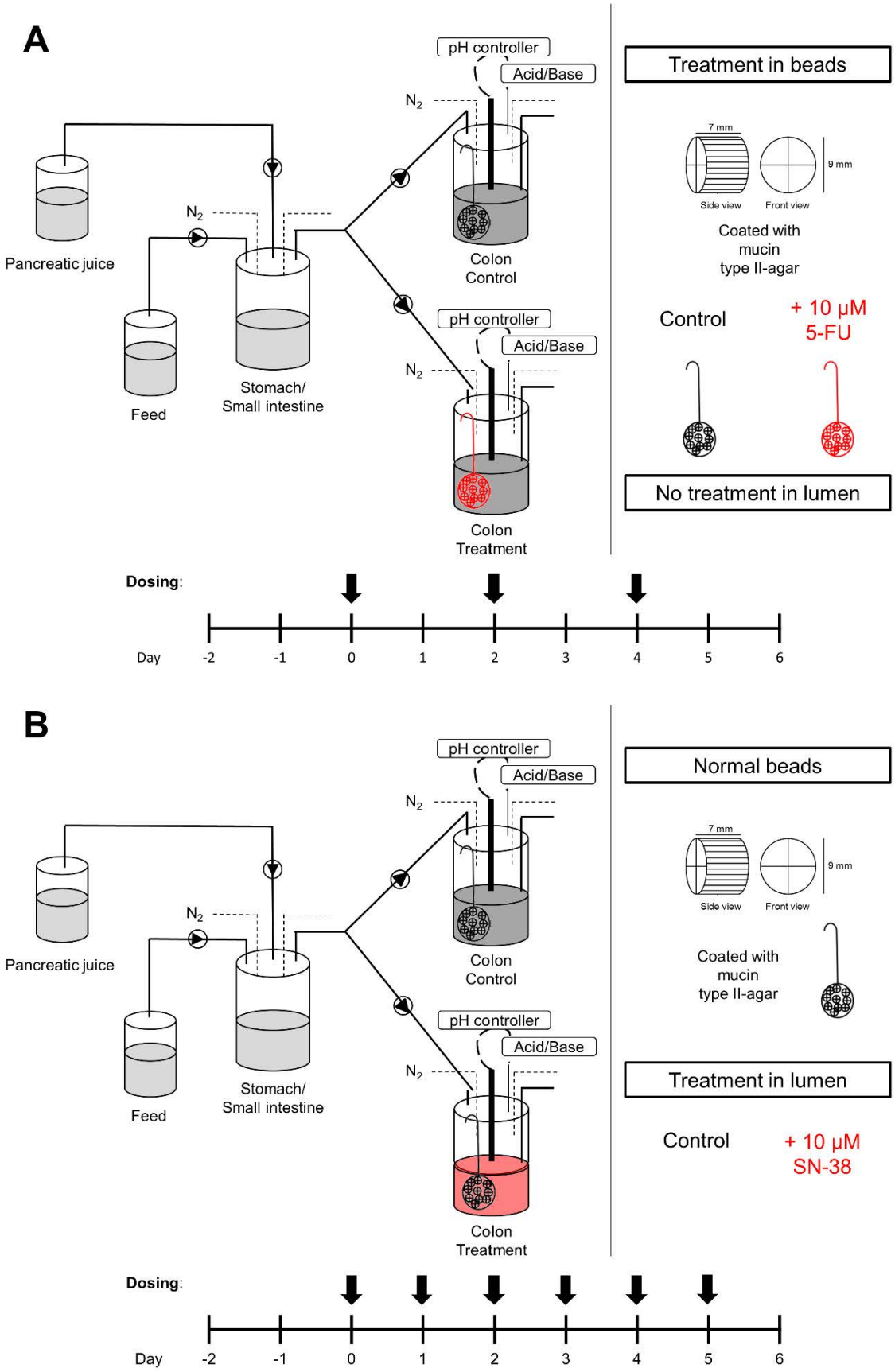


Figure 3.1 - Experimental set-up of A) M-SHIME with 5-FU and B) M-SHIME with SN-38. Arrows indicate the time of dosing.

2.4 DNA extraction

Total DNA was extracted from the pellet of 1 mL liquid samples or 0.25 g mucin-agar according to a protocol adapted from Vilchez-Vargas et al. (2013). Cells were lysed with 1 mL lysis buffer (100 mM Tris/HCl pH 8.0, 100 mM EDTA pH 8, 100 mM NaCl, 1 % (m/v) polyvinylpyrrolidone and 2 % (m/v) sodium dodecyl sulphate) and 200 mg glass beads (0.11 mm, Sartorius) in a FastPrep® 96 instrument (MP Biomedicals, Santa Ana, USA) for two times 40 s (1600 rpm). Following removal of glass beads by centrifugation (5 min at maximum speed), DNA was extracted from supernatant using a phenol–chloroform extraction. The DNA was precipitated at -20°C with 1 volume of ice-cold isopropyl alcohol and 0.1 volume of 3 M sodium acetate for at least 1 h. After removal of isopropyl alcohol by centrifugation (30 min, maximum speed), the DNA pellet was dried and resuspended in 100 µL 1x TE (10 mM Tris, 1 mM EDTA) buffer. The DNA samples were immediately stored at −20°C until further analysis. The quality of DNA samples was analyzed by gel electrophoresis (1.2 % (w/v) agarose) (Life technologies, Madrid, Spain). The DNA samples were diluted (1:10) for further analysis.

2.5 Microbial community analysis

2.5.1 Denaturing Gradient Gel Electrophoresis (DGGE)

The 16S rRNA gene region was amplified by means of PCR using the PRBA 338F-GC and 518R primers targeting the V3 region (Muyzer et al. 1993, Ovreas et al. 1997). The PCR program consisted of 10 min 95°C; 30 cycles of 1 min 94°C, 1 min of 53°C, 2 min of 72°C; and a final elongation for 10 min at 72°C. Amplification products were analyzed by gel electrophoresis (1.2 % (w/v) agarose). The DGGE was performed using the INGENY phorU System (Ingeny International BV, The Netherlands), based on the protocol of Muyzer et al. (1993). The PCR fragments were mixed with loading buffer (5:1) before loading onto 8 % (w/v) polyacrylamide gels with denaturing gradients ranging from 45 % to 60 % (where 100 % denaturant contains 7 M urea and 40 % formamide). The electrophoresis was run for 16 hours at 60°C and 120 V. Staining and analysis of the gels was performed as described previously (Boon et al. 2000). To process and compare the different gels, a homemade marker of different PCR fragments was loaded on each gel (Boon et al. 2002). The normalization and analysis of the DGGE gel patterns was carried out with the BioNumerics software 5.10 (Applied Maths, Sint-Martens-Latem, Belgium). The calculation of the similarity matrix was based on the Pearson correlation coefficient, and clustering was performed with the unweighted pair-group average method algorithm (UPMA).

2.5.2 16S rRNA gene Illumina MiSeq sequencing

Illumina amplicon sequencing was performed by LGC Genomics (Berlin, Germany) on the MiSeq platform. The V3-V4 region of the 16S rRNA gene was amplified using primers derived from Klindworth et al. (2013): 341F (NNNNNNNNNTCCTACGGGNGGCWGCAG) and 785R (NNNNNNNNNNTGACTACHVGGGTATCTAAKCC). The PCR mix included 1 µl of DNA extract, 15 pmol of both the forward and reverse primer in 20 µL of MyTaq buffer containing 1.5 units MyTaq DNA polymerase (Bioline) and 2 µl of BioStabII PCR Enhancer (Sigma). For each sample, the forward and reverse primers had the same unique 10-nt barcode sequence. The PCR program consisted of 2 min 96°C predenaturation and 20 cycli of 96°C for 15 s, 50°C for 30 s, 70°C for 90 s. Next, ~20 ng amplicon DNA of each sample was pooled for up to 48 samples carrying different barcodes. The amplicon pools were purified with one volume AMPure XP beads (Agencourt) to remove primer dimer and other small mispriming products, followed by an additional purification on MinElute columns (Qiagen). Finally, about 100 ng of each purified amplicon pool DNA was used to construct Illumina libraries by means of adaptor ligation using the Ovation Rapid DR Multiplex System 1-96 (NuGEN). Illumina libraries were pooled and size selected by preparative gel electrophoresis. Sequencing was done on an Illumina MiSeq using v3 Chemistry (Illumina). To assess the sequencing quality a mock community was included in triplicate in the sequencing run (error rate = 0.183 %).

The Mothur software package (v.1.33.3), and guidelines developed by Schloss et al. (2011) were used to process Illumina data. Forward and reverse reads were assembled into contigs and ambiguous contigs or contigs with divergent lengths were removed. The number of unique sequences was determined and these were aligned to the Mothur-reconstructed SILVA Seed alignment (v123). Sequences not aligning within the region targeted by the primer set or sequences with homopolymer stretches with a length higher than 12 were removed. Sequences were pre-clustered together within a distance of 1 nucleotide per 100 nucleotides. These cleaned-up and preclustered sequences were checked for Chimera's (with Uchime)(Edgar et al. 2011). The sequences were classified using RDP (Ribosomal Database Project) release 14 and a naive Bayesian classifier (Wang's algorithm). All sequences that were classified as Eukaryota, Archaea, Chloroplasts and Mitochondria were removed. If sequences could not be classified at all (even not at (super)Kingdom level) they were removed. The operational taxonomic units (OTUs) were clustered with an average linkage and at the 97 % sequence identity. Samples with less than 1000 reads per sample were not used for further analysis.

The sequences reported in this paper have been deposited in the European Nucleotide Archive (ENA) database (Accession number LT800946-LT802885).

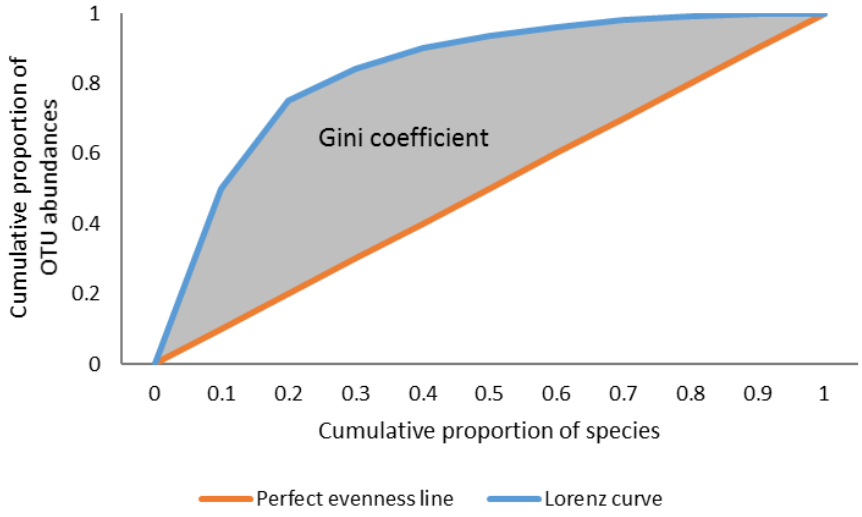
--Information box--

Studying the microbial community composition

Multiple diversity parameters can be used to describe a microbial community. These parameters can be calculated based on DGGE or Illumina amplicon sequencing data. Two main levels of diversity can be considered: α-diversity and β-diversity (Whittaker 1972).

α-diversity

The α-diversity is a measure for the diversity **within one sample** and multiple parameters can be used to quantify this α-diversity (Marzorati et al. 2008). **Richness** is a measure for the number of species present in a sample. For DGGE, the species richness corresponds with the number of bands. For amplicon sequencing, the species richness corresponds with the number of OTUs. **Evenness** is a measure for community organization. It is based on the difference in relative abundance of different species. Perfect evenness is defined as a community for which all species are present at the same abundance. Evenness can be quantified by the **Gini coefficient**, which is the surface between the Lorenz curve and the perfect evenness line (Box Figure 1) and can have values between 0 and 1. High Gini coefficients correspond to uneven communities and low Gini coefficients to even communities.



Box Figure 1: The Gini coefficient is a measure for the evenness of a community. The higher the Gini coefficient, the lower the evenness.

A more uniform way to describe diversity, is by using the **Hill numbers** (Hill 1973). Each Hill number represents a different level of diversity. Hill number **order 0 (H0)** is the richness of a sample, *i.e.* the number of OTUs in a sample. Hill number **order 1 (H1)** is the exponential of the Shannon index and takes not only into account richness, but also evenness. Hill number **order 2 (H2)** is the inverse of the Simpson index and takes into account evenness to an even higher degree.

β-diversity

The β-diversity is a measure for the diversity **between different samples**. Different **distance matrices** can be used to assess the β-diversity, such as Pearson correlation coefficient and Bray-Curtis dissimilarities. Based on these distance matrices, **clustering** of the samples can be done to check which samples are more similar to each other, for example via unweighted pair-group average method algorithm (UPMA) for DGGE data. **Ordination plots** can be used to visualize the similarities between samples in a 2D plot, based on the distance matrix. Non-metric Multidimensional Scaling (**NMDS**) is a non-parametric ordination method for which the visualization is optimized by reducing the 'stress', which represents the difference between the visualization and the actual distance matrix. However, statistical significance of differences between two groups is based on the complete distance matrix and can be determined by for example by Permutational ANOVA (**PERMANOVA**). To quantify the **community variation**, the distance to the centroid (= mean of a group) can be calculated for each sample based on the distance matrix. This is a measure for the within-group dispersion i.e. how variable samples are within a certain group.

2.6 Statistical analysis

All statistical analyses were performed in R (version 3.3.2).

Statistical inference on the 5-FU and SN-38 treatment effect on the SCFA concentration was performed by spline regression. Natural splines were fitted for each group (control and treatment) to the scaled and centered temporal data because these provide more stable estimates at the boundary time points (James et al. 2014). Knots were fixed at the 33.3 % and 66.6 % quantiles. Model parameter estimation was performed by the ordinary least squares method, resulting in model residuals that were normal distributed and did not exhibit temporal autocorrelation. Due to the presence of moderate heteroscedasticity in the model residuals, robust White heteroscedasticity-consistent standard errors (vcovHC function, sandwich v2.3-4 package) were used in the statistical inference on the treatment effect (type II ANOVA).

The packages phyloseq (McMurdie and Holmes 2013) and vegan (Oksanen 2016) were used for microbial community analysis. Heatmaps were generated with the pheatmap package and order-based Hill's numbers (Hill 1973) were calculated. If the data were normally distributed (tested with Shapiro-Wilk test) and homoscedastic (tested with Levene test), differences in Hill's numbers were defined via ANOVA and Tukey as post-hoc test; if not, Kruskall Wallis test with Tukey post-hoc testing was used. Non-metric multidimensional scaling (NMDS) plots of the bacterial community data were created based on the Bray-Curtis distance measures. Significant differences were identified by means of Permutational ANOVA (PERMANOVA) using the *adonis* function (vegan).

3. Results

3.1 Effect of 5-FU on the metabolic activity in the gut

The effect of 5-FU (at 10 µM in the mucosal environment) on the gut microbiome of 6 healthy donors was investigated using a M-SHIME® harboring both luminal and mucosal microbiota (Figure 3.1A). SCFA analysis showed interindividual differences and the total luminal SCFA concentration before the first treatment ranged from 25.5 µM to 39.5 µM between the six donors. Overall, there is no significant difference between treatment with 5-FU and control behavior through time (p=0.18). Although, for donor 1 and donor 2, total SCFA concentrations started increasing after the second 5-FU treatment, compared with the control, with a final increase of 113 % and 76 % respectively at day 6 (Figure 3.2). The same trends (i.e., an increase for donor 1 and 2 and no effect for all other donors), were observed for the total mucosal SCFA concentration (Supplementary Figure 3.1). Further, 5-FU did not have any effect on the relative proportions of acetate, propionate, butyrate or branched SCFA (Supplementary Figure 3.2). For donor 1 and 2, the experimental set-up also allowed the inclusion of a distal colon region (pH 6.6-6.9). However, we observed no effect of 5-FU on the SCFA production (Supplementary Figure 3.3)

3.2 Effect of 5-FU on the gut microbial profile

To investigate the effect of 5-FU on the composition of both luminal and mucosal microbiota, both DGGE and Illumina sequencing of the 16S rRNA gene were performed for all samples at day 0 and day 6.

Clustering analysis of DGGE profiles showed prominent interindividual variability, but no significant impact of 5-FU treatment. Even after 6 days of treatment, profiles still clustered together for each donor with similarities between control and treatment ranging from 79-98 % for luminal samples and 93 % to 99 % for mucosal samples, except for donor 2 who had only 70 % similarities. Hence, there was no clear shift in the microbial profile for all donors, only minor differences between control and 5-FU treated samples could be observed (Supplementary Figure 3.4).

Illumina sequencing showed that all samples were dominated by *Proteobacteria*, *Bacteroidetes* and *Firmicutes*. At the genus level, *Escherichia*/*Shigella*, *Bacteroides*, *Veillonella* and *Clostridium cluster XIVa* were most abundant (Figure 3.3 and Supplementary Figure 3.5). Also NMDS plots showed no significant difference between control and 5-FU treatment (p=0.75) and treatment condition only explained 2.7 % of the variation of the microbiome after treatment (based on Bray–Curtis dissimilarities on OTU level) (Figure 3.4A).

For all samples, donor individuals and sampling time point explained most of the variation, respectively 38.9 % and 14.8 % (p=0.0001 and p=0.0001) (Table 3.1).

At the genus level, the 5-FU treatment showed a trend in increased relative abundance of *Bacteroides* from 24.4 ± 13.2 % to 41.1 ± 11.6 % (p=0.065) and decreased abundance of *Escherichia*/*Shigella* from 19.7 ± 17.1 % to 8.9 ± 7.2 % (p= 0.23) in the lumen for 5 out of 6 donors. In the mucus fraction, these shifts could not be observed. In contrast, 5-FU did not influence the diversity in the lumen at the end of treatment, but did increase the diversity (first and second order Hill numbers) in the mucus (p=0.010 and p=0.055 respectively), indicating a higher evenness and diversity (Supplementary Figure 3.6). In general, mucus samples had a higher diversity than luminal samples (p-values for Hill numbers: p=0.058, p=0.0032 and p=0.0039) (Supplementary Figure 3.6). Only for donor 2, a changed mucus bacterial profile was observed, with increases in *Anaeroglobus*, *Roseburia* and *Parabacteroides* (Figure 3.3 and Supplementary Figure 3.5).

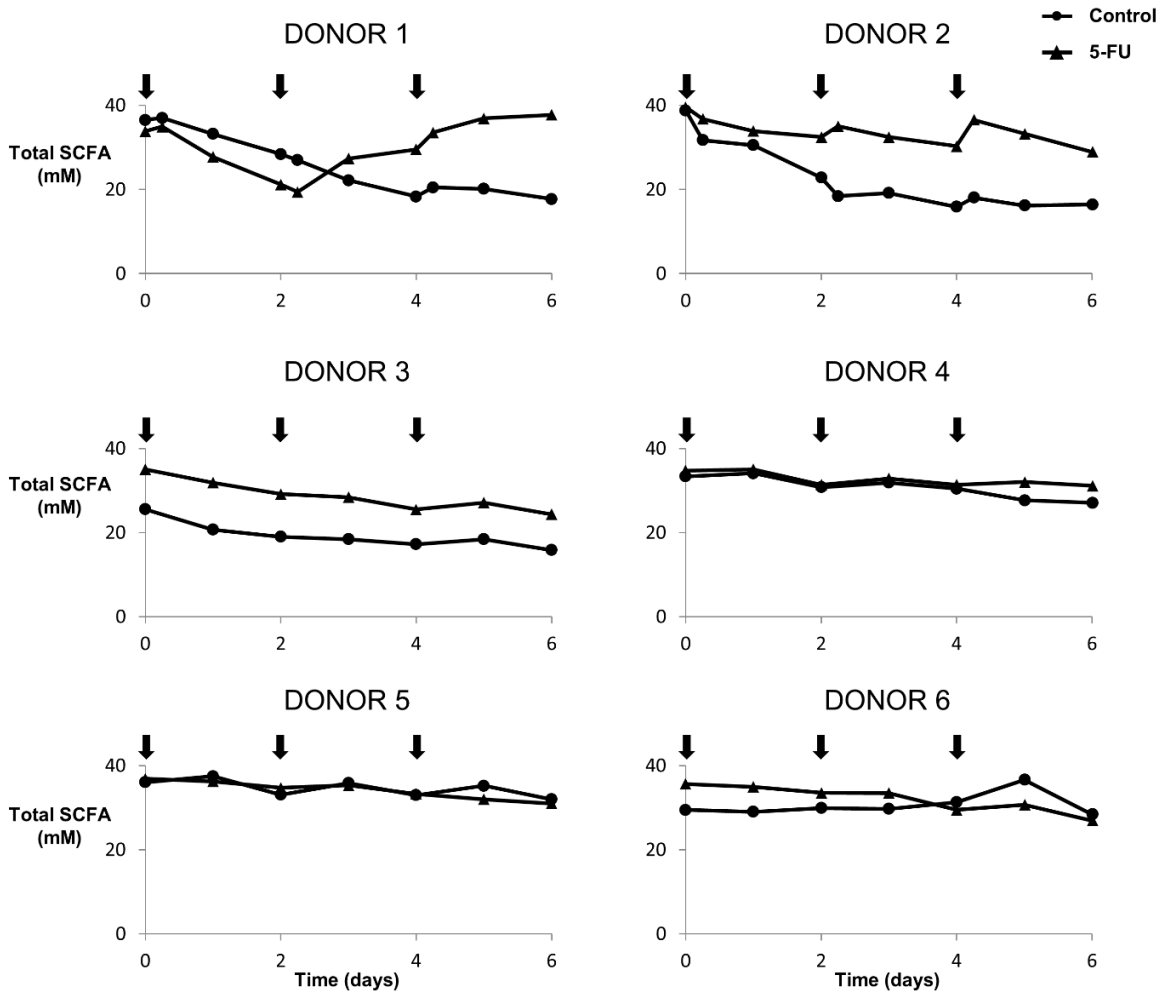


Figure 3.2 - At 10 µM in the mucosal environment of the M-SHIME, 5-FU increases luminal total short chain fatty acid concentrations for donor 1 and 2 and has no effect for all other donors. Arrows indicate the time of dosing.

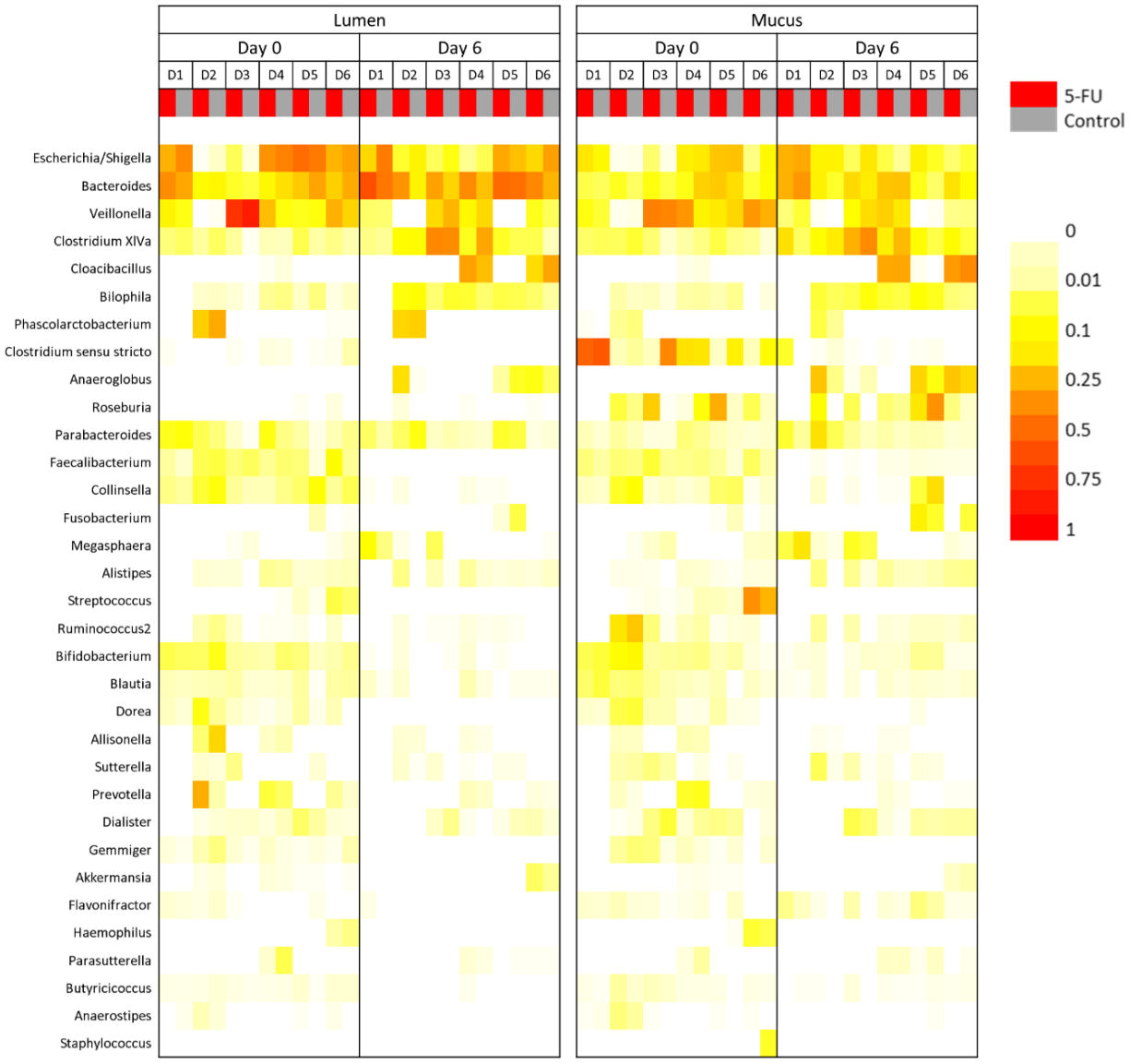


Figure 3.3 - Heatmap representing the most abundant genera (at least 0.1 % on average) showed no clear effect of treatment with 5-FU on the gut microbial composition of 6 healthy donors (D1-D6).

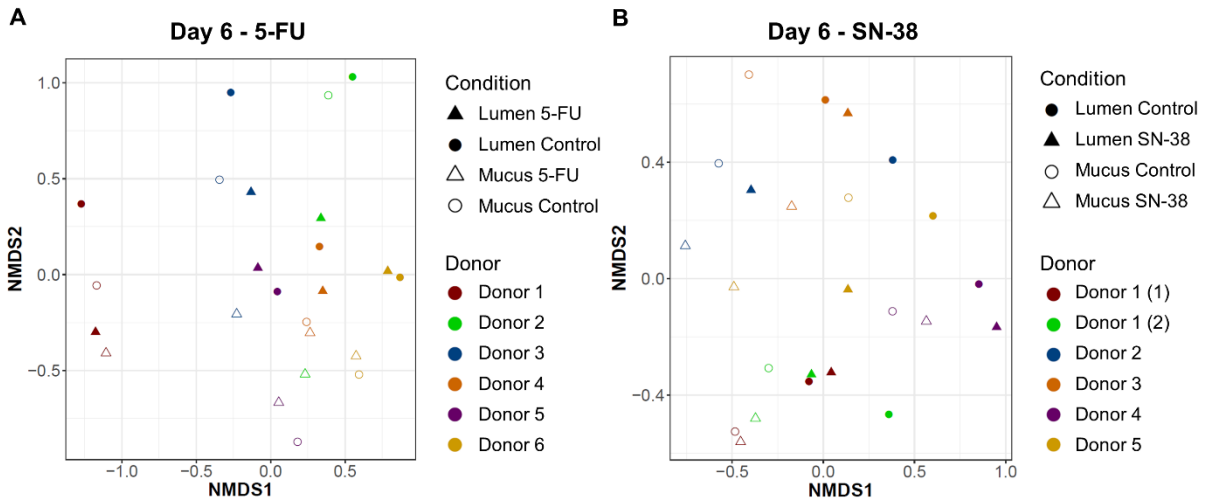


Figure 3.4 - NMDS plots based on Bray Curtis dissimilarities of Illumina sequencing data at day 6 (after treatment) showed large interindividual variability, but no clear effect of the treatment with A) 5-FU or B) SN-38.

Table 3.1 - p-values and R^2 for different confounding factors based on Bray Curtis dissimilarities of the Illumina sequencing data (significant values are indicated in italic).

5-FU		**Donor**	**Time**	**Lumen/ Mucus**	**Treatment**
All	p-value	*0.0001*	*0.0001*	*0.0071*	0.90
	R^2	*38.9 %*	*14.8 %*	*5.2 %*	1.2 %
Day 0	p-value	*0.0001*	NA	*0.033*	0.96
	R^2	*63.8 %*	NA	*8.7 %*	1.8 %
Day 6	p-value	*0.0001*	NA	0.16	0.75
	R^2	*70.1 %*	NA	6.5 %	2.7 %
SN-38		**Donor**	**Time**	**Lumen/ Mucus**	**Treatment**
All	p-value	*0.0097*	*0.0001*	*0.0014*	0.72
	R^2	*17.7 %*	*30.3 %*	*9.0 %*	1.4 %
Day 0	p-value	*0.0001*	NA	*0.0028*	0.77
	R^2	*54.2 %*	NA	*13.7 %*	2.7 %
Day 6	p-value	*0.0001*	NA	*0.0001*	0.31
	R^2	*44.3 %*	NA	*24.0 %*	5.1 %

3.3 Effect of SN-38 on the metabolic activity in the gut

The effect of SN-38 (the active metabolite of irinotecan) (at 10 µM in the luminal environment) on the gut microbiome of 5 healthy donors was investigated using a M-SHIME with proximal colon vessels (Figure 3.1B). Interindividual differences in the total luminal SCFA concentration before the first treatment ranged from 18.3 µM to 45.9 µM between the five

donors. Overall, there is no significant difference between treatment with SN-38 and control behavior through time (p=0.85) (Figure 3.5). A similar trend (no effect of SN-38) was observed for the total mucosal SCFA concentrations (Supplementary Figure 3.8). Also for the relative luminal concentrations of acetate, propionate, butyrate and branched SCFA, no impact of SN-38 was detected (Supplementary Figure 3.9). For donor 1 (in duplicate), the experimental set-up allowed the inclusion of a distal colon region (pH 6.6-6.9). However, we observed no effect of SN-38 had on the SCFA levels of the distal colon microbiota (Supplementary Figure 3.10). Similarly, the experimental set-up also allowed running a luminal-SHIME system (without mucus compartment). However, again no effect on SCFA production was observed, excluding the fact that the mucus layer protects the microbiota against the SN-38 (Supplementary Figure 3.11).

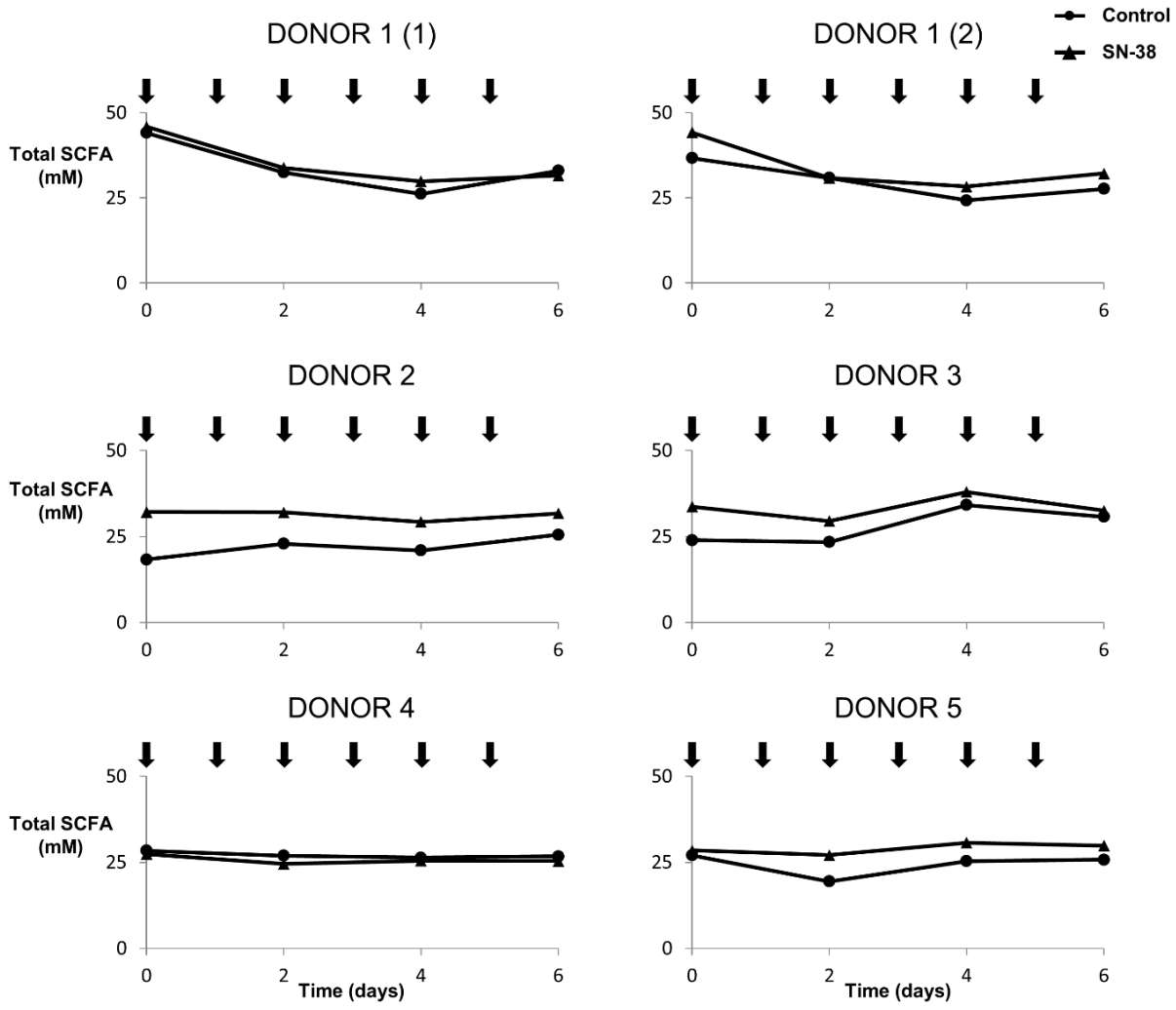


Figure 3.5 - At 10 µM in the luminal part of the M-SHIME, irinotecan (SN-38) has no effect on luminal total short chain fatty acid concentrations. Arrows indicate the time of dosing.

3.4 Effect of SN-38 on the gut microbial profile

To investigate the effect of SN-38 on the composition of both luminal and mucosal microbiota, DGGE and Illumina sequencing of the 16S rRNA gene were performed for all samples at day 0 and day 6.

Clustering analysis of DGGE profiles showed large interindividual variability, but no effect of SN-38 treatment was observed. After 6 days of treatment microbial profiles still clustered together for each donor with similarities ranging from 94 to 98 % for luminal samples (even higher than at the start) and 68 to 92 % for mucosal samples. There were no clear shifts in the microbial profile of all 5 donors and only minor differences between control and SN-38 treated samples could be observed (Supplementary Figure 3.12).

Illumina sequencing showed that all samples were dominated by *Proteobacteria*, *Bacteroidetes* and *Firmicutes*. At the genus level, *Escherichia*/*Shigella*, *Bacteroides*, *Veillonella* and *Clostridium cluster XIVa* were most abundant (Figure 3.6 and Supplementary Figure 3.13). The NMDS plots demonstrated no significant difference between control and SN-38 treatment (p=0.31) and the treatment condition only explained 5.1 % of the variation of the microbiome after treatment (based on Bray-Curtis dissimilarities on OTU level) (Figure 3.4B). For all samples, donor individuals and sampling time point explained most of the variation, respectively 17.7 % and 30.3 % (p= 0.0097 and p=0.0001) (Table 3.1).

No effect of SN-38 on specific genera or diversity indices were noticed for all donors, but some donor-specific changes of relative abundances of some genera could be observed. For donor 2 and 3, an increase in *Cloacibacillus* was observed (from respectively 1.4 to 14.6 % and 0 to 22.4 %) in presence of SN-38 in the lumen, compared with the control. For donor 3 and donor 5, *Alistipes* increased in the lumen in presence of SN-38 compared with the control (from 0.4 to 9.4 % and 0.09 to 7.8 % respectively). In the mucus, an increase in *Roseburia* for donor 1 (in duplicate) and donor 2 was observed in presence of SN-38 compared with the control (from 9.9 to 24.9 %, 2.4 to 25.1 % and 2.1 to 18.5 % respectively) (Figure 3.6 and Supplementary Figure 3.13). Similar to 5-FU, mucosal samples showed a higher evenness compared with luminal samples (Hill number order 1, p=0.0094) (Supplementary Figure 3.6).

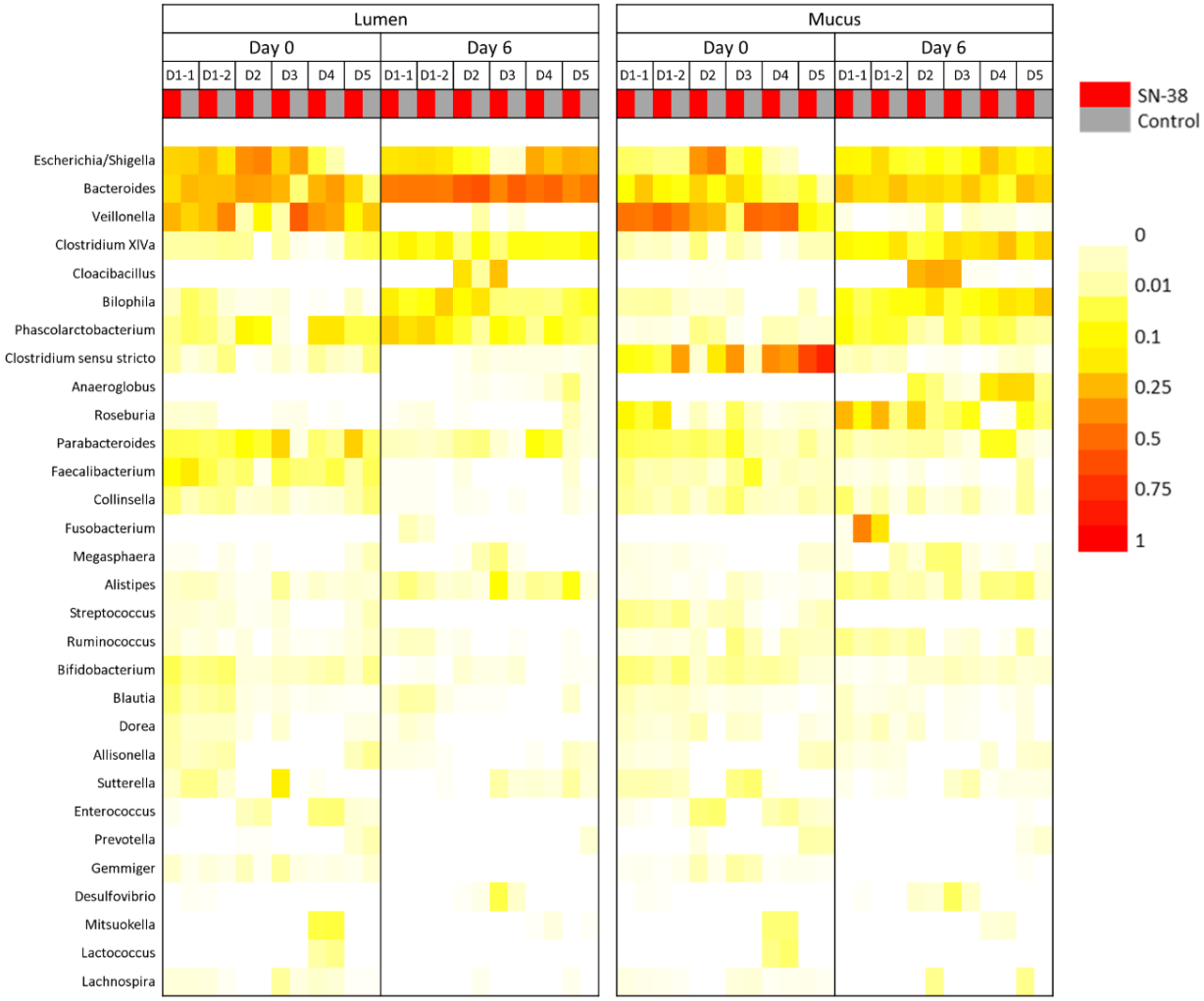


Figure 3.6 - Heatmap representing the most abundant genera (at least 0.1 % on average) showed no clear effect of treatment with SN-38 on the gut microbial composition of 5 healthy donors (D1-D5).

4. Discussion

Patients often have to deal with several side effects from cancer treatment, of which gastrointestinal mucositis is one of the most debilitating. The major symptoms are inflammation and ulceration of the gastrointestinal tract accompanied with diarrhea. Gut microbiota are more and more believed to play a role in the etiology and severity of mucositis, but the direct effect of chemotherapy on gut microbiota is not yet clear. In this study, we show that 5-FU and SN-38 only have a limited impact on colon microbial functionality and composition in an *in vitro* model mimicking both the luminal and mucosal gut microbiota.

5-FU was added solely to the mucosal part of the M-SHIME® as it reaches the gut mainly via the blood and gut mucosa. Plasma concentrations of 5-FU can reach some hundreds of µM in cancer patients, for example with a bolus injection with 5-FU, but they rapidly drop to 15-30 µM after 30 minutes and to 0 µM after 2 h (Casale et al. 2004, Kosovec et al. 2008), due

to the short half life time (6-22 min) of 5-FU (Bocci et al. 2000). In contrast, for a continuous infusion with 5-FU, the plasma concentrations will be much lower, ranging from 3 to 10 µM, but are steady for a longer time period (24 h) (Joulia et al. 1999, Takimoto et al. 1999). As we wanted to investigate a long term dosing, we chose to dose at 10 µM 5-FU in the mucus layer, which is in close contact with the blood circulation.

The active metabolite of irinotecan, SN-38, was added to the luminal part of an M-SHIME® as it reaches the colon via the small intestine and through microbial synthesis, hydrolyzing biliary secreted, non-toxic SN-38G to the active compound SN-38 by β-glucuronidase activity (Takasuna et al. 1996). This also explains why feces levels of SN-38 are much higher than plasma levels. Considering that on average 1.2 % of the dose is excreted as SN-38 in the feces within 24 h (Sparreboom et al. 1998, Slatter et al. 2000), (an estimated average colon concentration of ~1-2 µM), and assuming a homogeneous distribution along the colon, a dose of 10 µM SN-38 was used in the luminal part of the M-SHIME.

Neither 5-FU nor SN-38 had a significant impact on the functionality or the composition of the M-SHIME community. Only some donor-specific changes could be observed: increases in *Bacteroides*, *Cloacibacillus*, *Alistipes* and *Roseburia* and a decrease in *Escherichia/Shigella* after chemotherapeutic treatment. Our observations are in contrast with what was found in human trials analyzing stool samples after chemotherapy, namely an increase in *Escherichia* and a decrease in *Roseburia* and varying trends for *Bacteroides* (van Vliet et al. 2009, Zwielehner et al. 2011, Stringer et al. 2013, Montassier et al. 2014, Montassier et al. 2015) However, these studies used different chemotherapeutic agents and the use of antibiotics was not excluded. With regards to animal studies, an increase in *Escherichia* was shown in fecal samples of rats treated with 5-FU (Stringer et al. 2009c).

No shifts in the M-SHIME microbiome could be observed after 5-FU treatment, although our previous research on single species clearly showed a differential sensitivity effect amongst oral species towards the drug (Vanlancker et al. 2016) and the same trend was observed for gastrointestinal microbiota (Stringer et al. 2009c, Florez et al. 2016). For example, *Escherichia coli* and *Pseudomonas aeruginosa* were highly resistant to 5-FU whereas *Bifidobacteria* were much more sensitive (Stringer et al. 2009c, Florez et al. 2016, Vanlancker et al. 2016). Apparently, when present in a well-balanced ecosystem as the M-SHIME system, the impact of 5-FU is very low. For irinotecan on the other hand, 34 gut species are shown to be resistant till 330 µM irinotecan (Florez et al. 2016), although *in vivo* transformation of irinotecan to more toxic compounds as SN-38 had not been taken into account in this study. For both 5-FU and SN-38, however, we could not evaluate what the effect of chemotherapeutics would be on an unbalanced ecosystem, as in our study, stool samples from healthy donors (non-antibiotic exposed) were used as inoculum. Therefore, it may be interesting to investigate the effect of chemotherapeutic agents on an unbalanced gut microbial ecosystem, such as after antibiotic

treatment. As many patients will also receive antibiotics during their treatment, chemotherapeutics may therefore have a bigger impact on this unbalanced, less diverse microbiome.

Although we did not see a major direct impact of chemotherapeutic treatment (5-FU and SN-38) on the colon microbiota *in vitro*, both clinical and animal studies show that these chemotherapeutic treatments can have an impact on the gut microbiota. Rat studies with 5-FU and SN-38 have shown that there are clear shifts in the microbial composition after chemotherapy (Stringer et al. 2007, Touchefeu et al. 2014). We hypothesize, that host-microbe interactions or the presence of the host are needed to induce these changes *in vivo* and that there is only a minor direct effect of chemotherapy on the gut microbial ecosystem as such. This also suggests that the host is a major contributing element in the drug toxicity towards the gut microbiome. The stressed host environment can induce or aggravate microbial dysbiosis and indirectly increase gastrointestinal mucositis severity. Targeting the microbiome with probiotics could still be a good strategy to treat mucositis by supporting a homeostatic gut microbial ecosystem. Gut microbiota can protect the intestinal mucosa by regulating important pathways in mucositis such as TLR-NF-κB pathway, mucus layer, intestinal permeability, mucosal repair etc (Touchefeu et al. 2014).

In conclusion, 5-FU and SN-38 displayed a limited impact on microbial composition and functionality of a healthy *in vitro* M-SHIME® colon ecosystem. We know from clinical and animal studies that the microbiome changes upon treatment with chemotoxic agents and therefore we assume that these changes are primarily induced in presence of host cells. These modulation of the host cells and tissue can have a major impact on the gut microbiome resulting in dysbiosis that can further aggravate the mucositis process. This mechanism where disrupted host-microbe interactions under chemotherapeutic stress contribute to the process of mucositis, needs to be further investigated.

5. Acknowledgements

E.V. is a doctoral research fellow supported by the Bijzonder Onderzoeksfonds (Ghent University; BOF13/DOC/280). This research was funded by BOF17/GOA/032 from Ghent University. B.V. was funded by the Seventh Framework Programme (FP7/2011) under grant agreement n° 299169 (Mucositis Platform). A.S. was supported by a CAESIE: Connecting Australian-European Science and Innovation Excellence Priming Grant. The authors would like to thank Jana De Bodt and Janie Bourgeois for the technical support, Jo De Vrieze for the support in analyzing the Illumina data, Ruben Props for the statistical support and Rosemarie De Weirdt and Jo De Vrieze for their review of the manuscript.

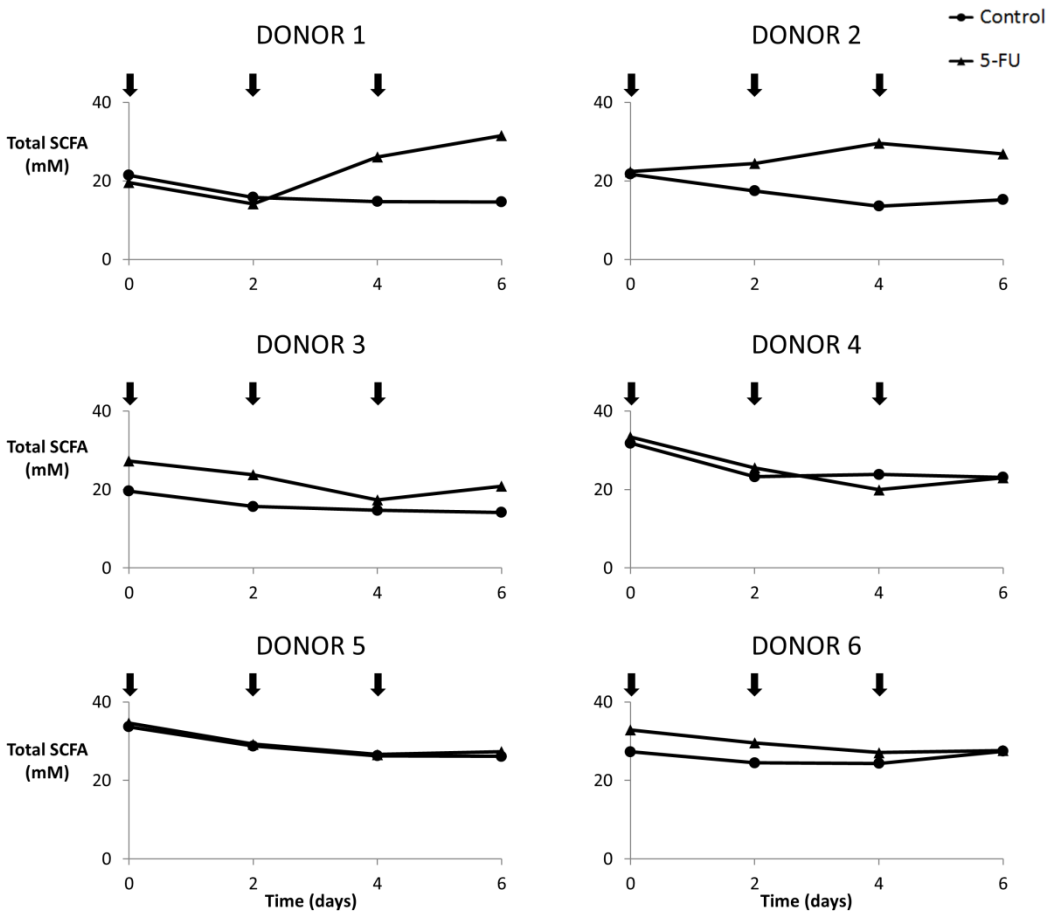
6. Supplementary information

Supplementary Table 3.1 - Abundance of major phyla (%), based on Illumina MiSeq sequencing of the 16S rRNA gene, for different types of samples in the M-SHIME runs with 5-FU (AV ±SD)

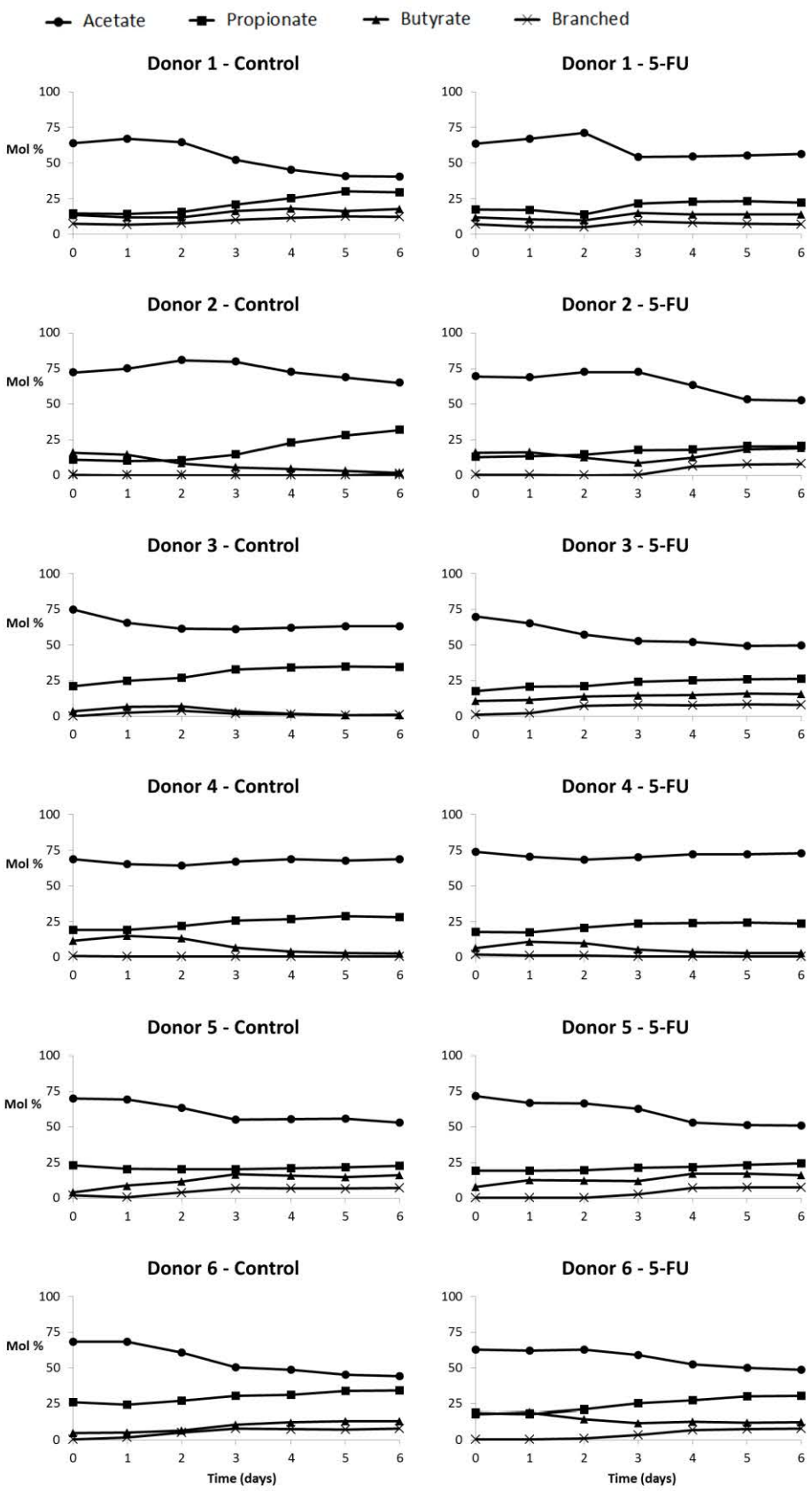
			Firmicutes	Bacteroidetes	Proteobacteria
Lumen	Day 0	Control	39,1 ± 31,9	23,6 ± 12,4	29,6 ± 22,0
		5-FU	41,8 ± 23,3	26,9 ± 14,1	26,4 ± 19,0
	Day 6	Control	28,5 ± 23,7	31,2 ± 15,3	30,3 ± 17,3
		5-FU	27,7 ± 19,6	46,1 ± 12,3	17,6 ± 9,9
Mucus	Day 0	Control	67,0 ± 20,8	11,3 ± 9,5	15,2 ± 15,4
		5-FU	71,3 ± 13,2	11,1 ± 6,3	12,6 ± 7,9
	Day 6	Control	48,1 ± 16,8	16,5 ± 11,3	18,9 ± 11,5
		5-FU	47,9 ± 12,8	21,7 ± 8,8	16,7± 9,3

Supplementary Table 3.2 - Abundance of major phyla (%), based on Illumina MiSeq sequencing of the 16S rRNA gene, for different types of samples in the M-SHIME runs with SN-38 (AV ±SD)

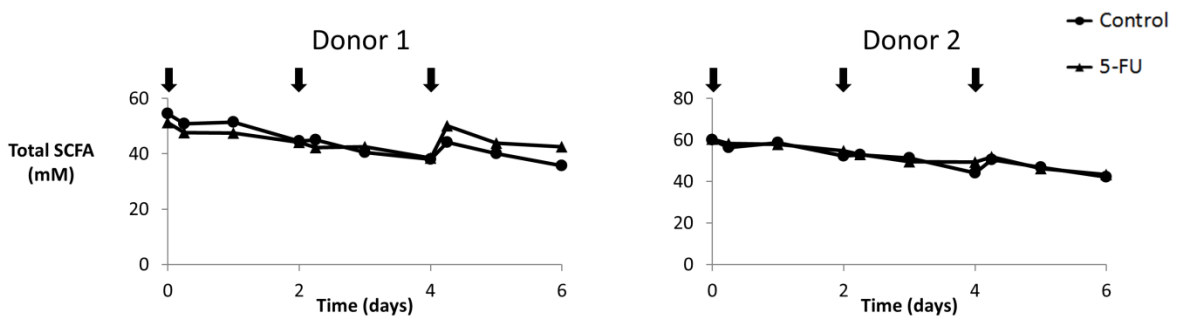
			Firmicutes	Bacteroidetes	Proteobacteria
Lumen	Day 0	Control	46,9 ± 15,7	23,2 ± 14,7	27,0 ± 16,4
		SN-38	36,5 ± 18,2	33,2 ± 9,3	27,0 ± 11,9
	Day 6	Control	23,1 ± 9,0	53,3 ± 7,6	23,2 ± 10,9
		SN-38	17,8 ± 8,1	51,2 ± 5,9	24,6 ± 9,6
Mucus	Day 0	Control	75,4 ± 19,9	10,7 ± 8,2	11,3 ± 16,8
		SN-38	73,0 ± 17,8	14,3 ± 7,8	10,1 ± 12,6
	Day 6	Control	44,8 ± 15,8	19,3 ± 2,9	23,5 ± 9,1
		SN-38	44,2 ± 4,6	21,6 ± 4,4	21,4 ± 6,5



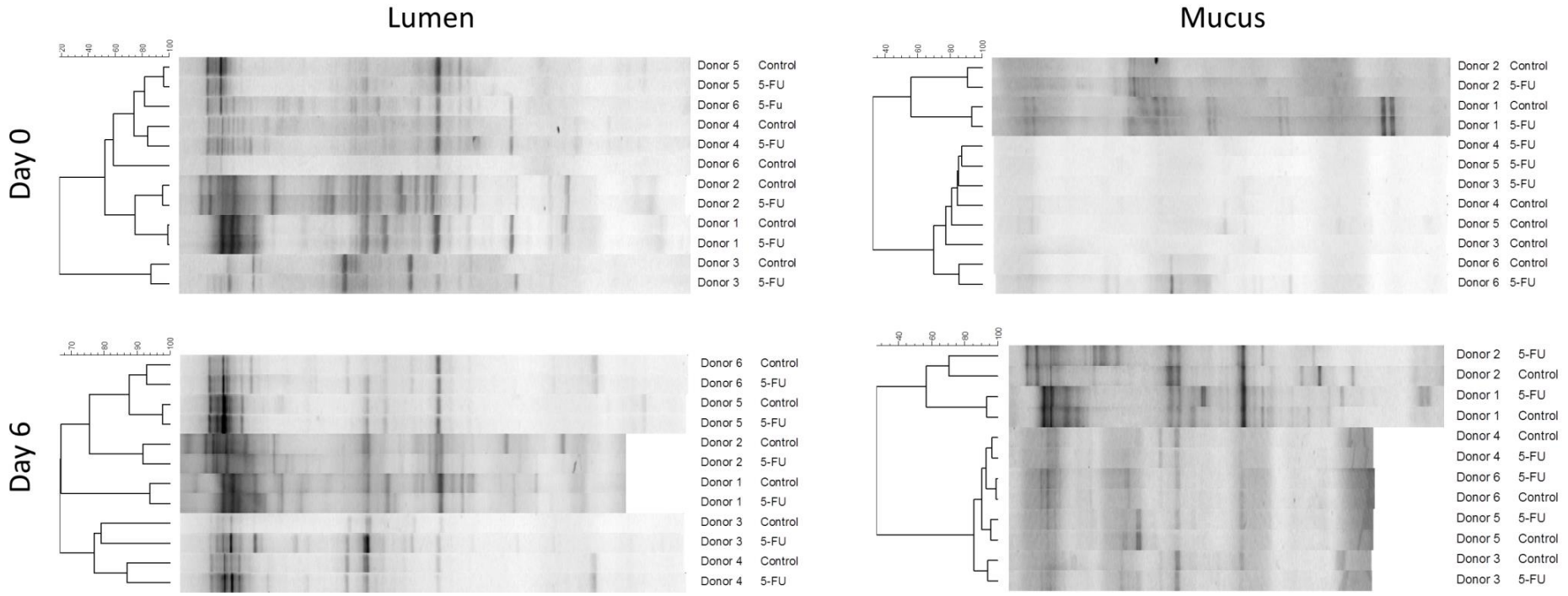
Supplementary Figure 3.1 - At 10 µM in the luminal part of the M-SHIME, 5-FU has limited effect on mucosal total short chain fatty acid concentrations (arrows indicate the time of dosing).



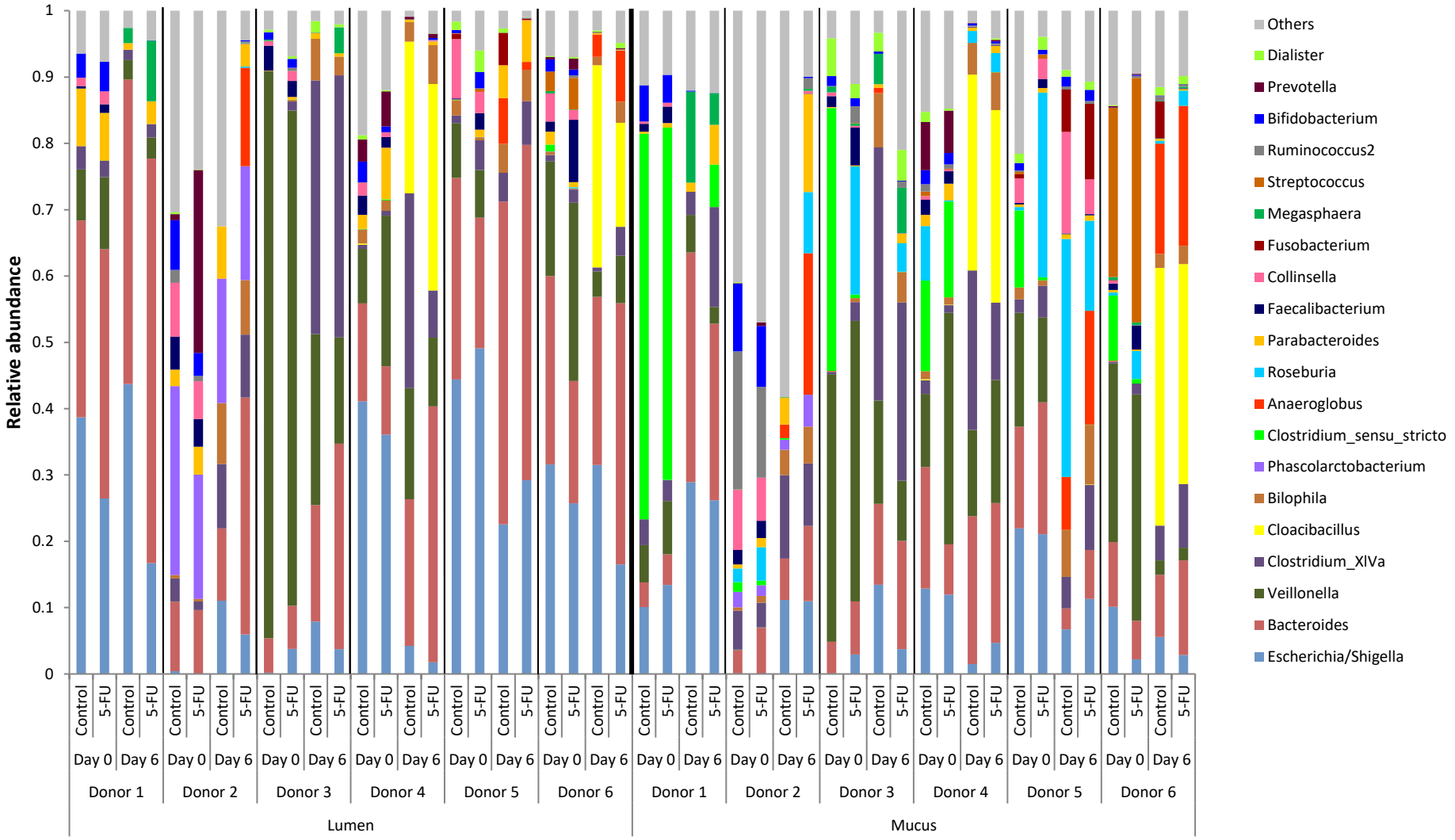
Supplementary Figure 3.2 - At 10 µM in the luminal environment of the M-SHIME, 5-FU has no effect on luminal relative short chain fatty acid concentrations.



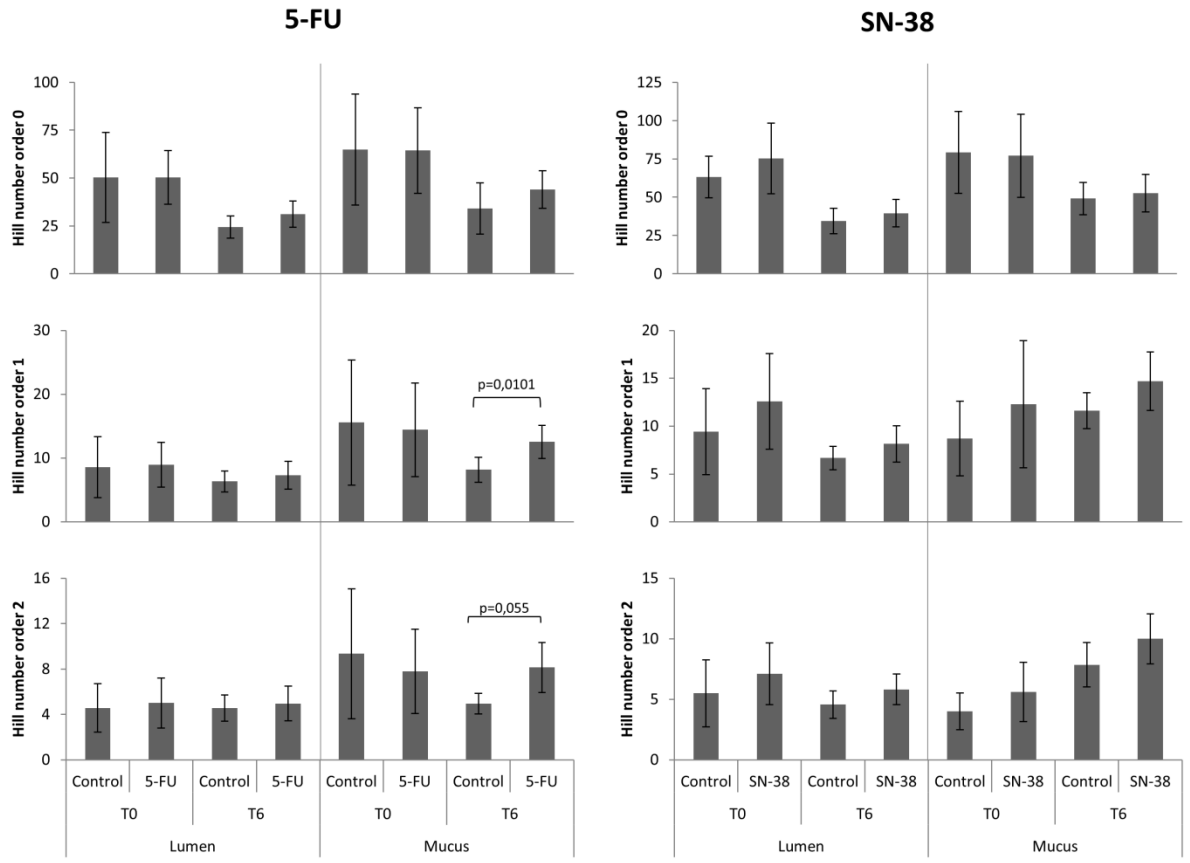
Supplementary Figure 3.3 - At 10 µM in the luminal environment of the distal part of the M-SHIME, 5-FU has no effect on luminal relative short chain fatty acid concentrations.



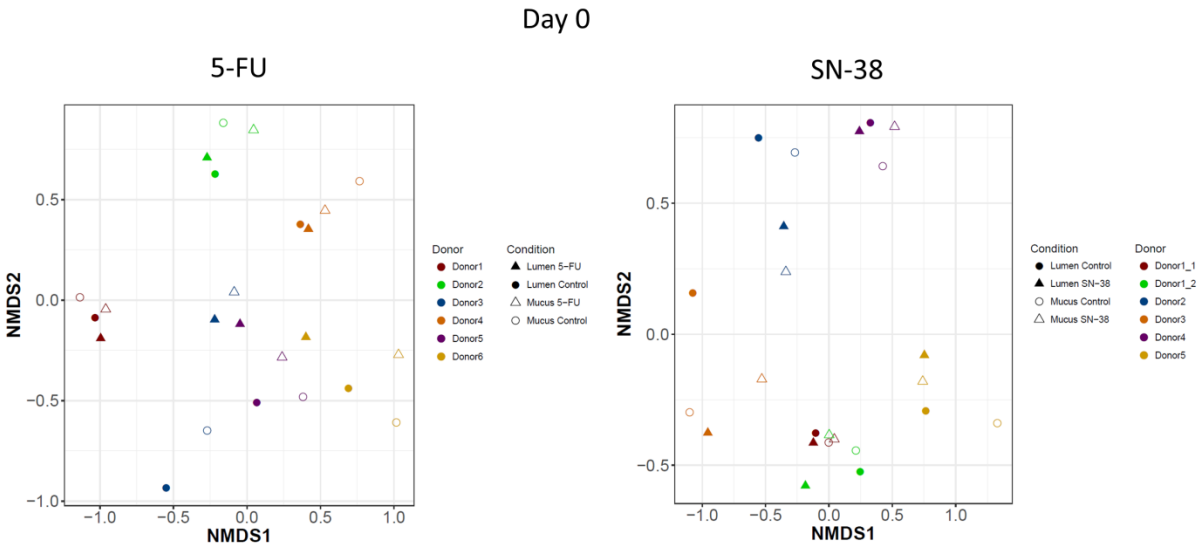
Supplementary Figure 3.4 - At 10 µM in the mucosal part of the M-SHIME, 5-FU has no clear effect on both the luminal and the mucosal microbial composition as shown with DGGE.



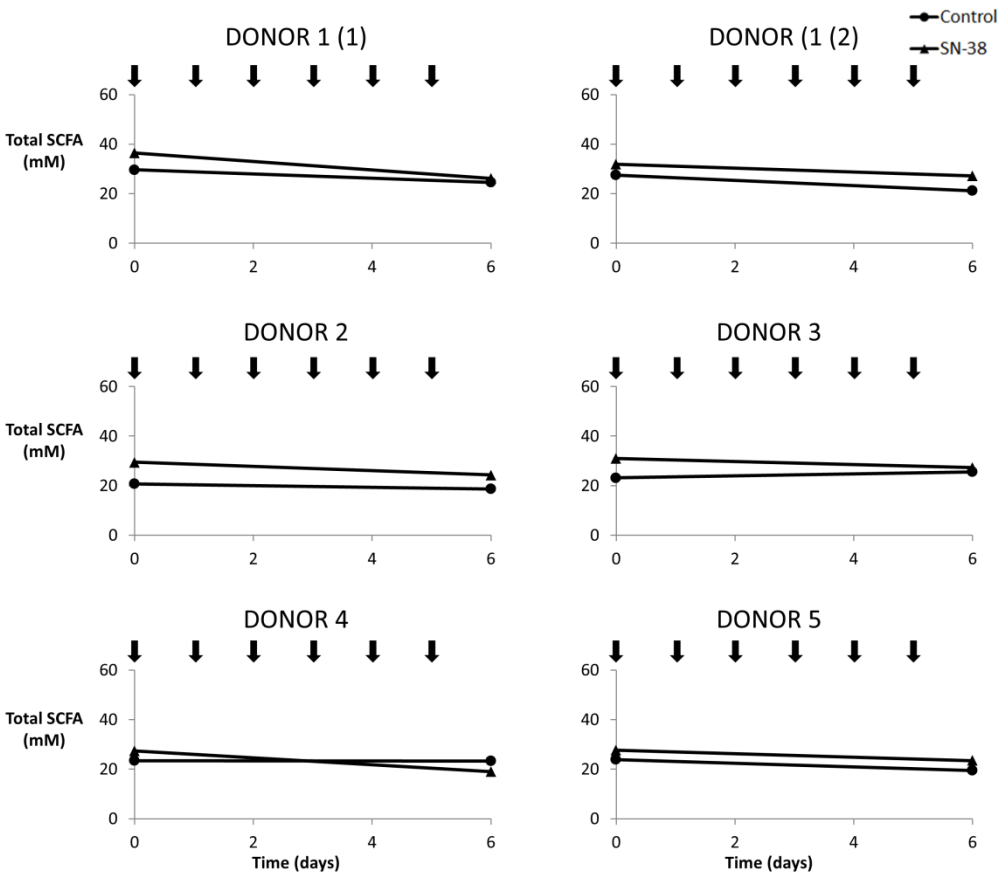
Supplementary Figure 3.5 - Relative abundances of the 20 most abundant genera of the microbial community of the M-SHIME run with 5-FU based on Illumina Miseq sequencing of the 16S rRNA gene.



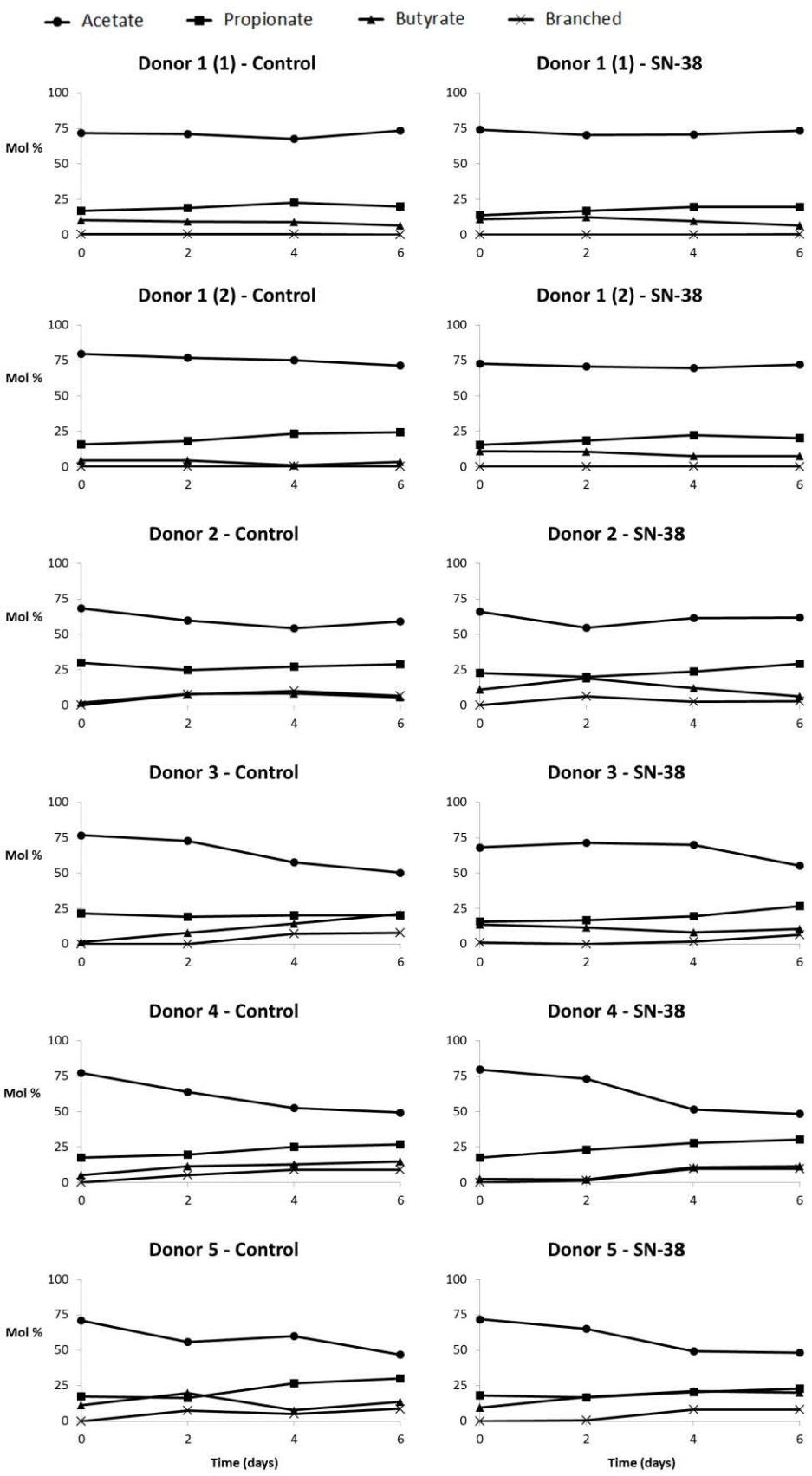
Supplementary Figure 3.6 - Hill numbers based on Illumina sequencing of the 16S rRNA gene as a measure for diversity. Order 0 displays species richness, order 1 the exponential of Shannon's entropy index and order 2 the inverse of Simpson's concentration index.



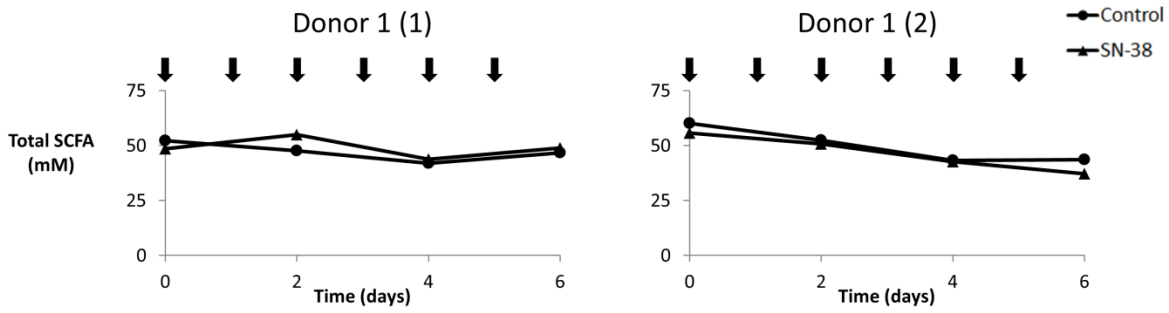
Supplementary Figure 3.7 - NMDS plots based on Bray Curtis dissimilarities of Illumina data on day 0 showed high interindividual variability.



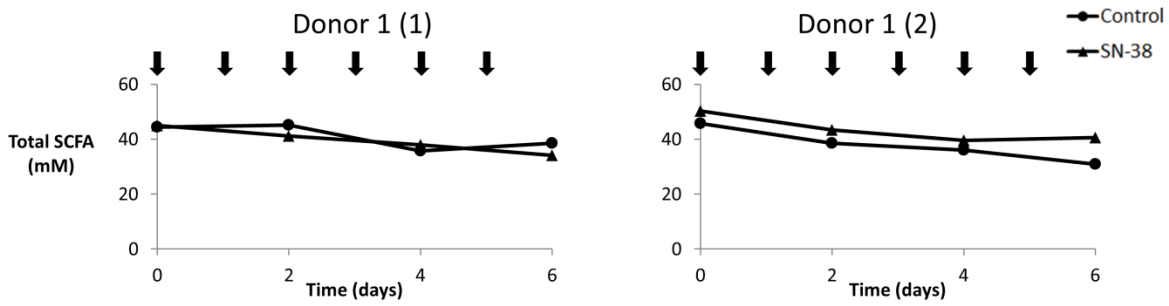
Supplementary Figure 3.8 - At 10 µM in the luminal part of the M-SHIME, irinotecan (SN-38) has no effect on mucosal total short chain fatty acid concentrations (arrows indicate the time of dosing).



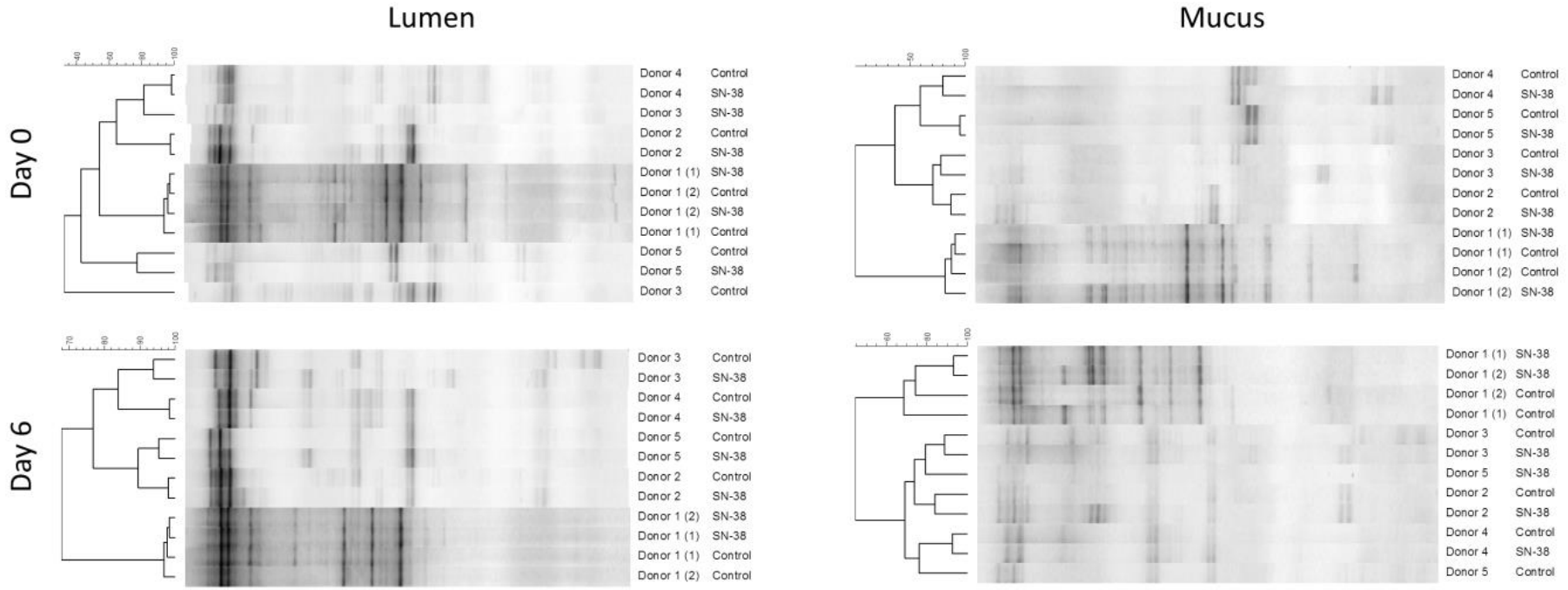
Supplementary Figure 3.9 - At 10 µM in the luminal part of the M-SHIME, irinotecan (SN-38) has no effect on luminal relative short chain fatty acid concentrations.



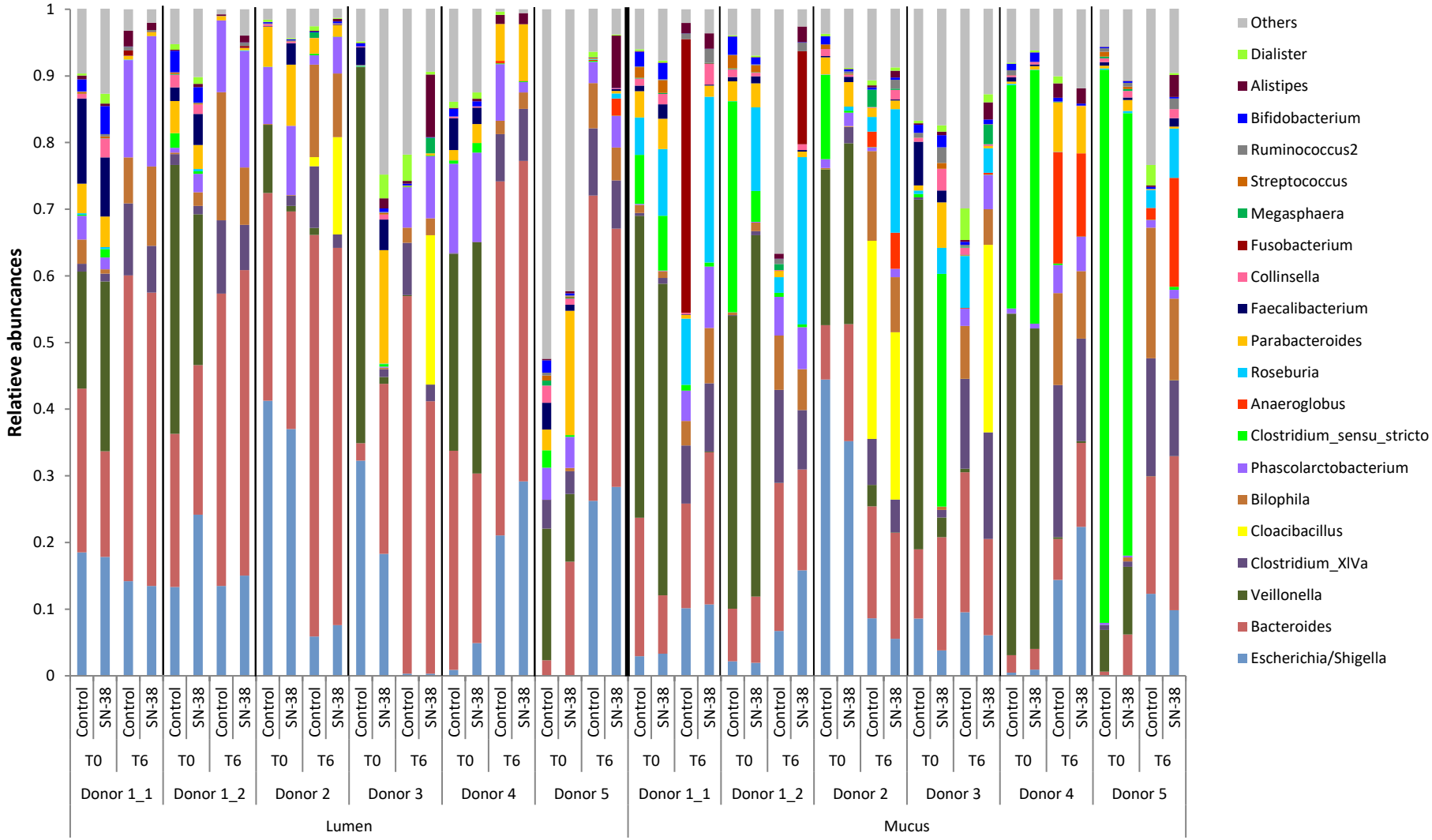
Supplementary Figure 3.10 - At 10 µM in the luminal part of the distal environment of the M-SHIME, irinotecan (SN-38) has no effect on luminal total short chain fatty acid concentrations.



Supplementary Figure 3.11 - At 10 µM in the luminal part of the proximal environment of the L-SHIME, irinotecan (SN-38) has no effect on luminal total short chain fatty acid concentrations.



Supplementary Figure 3.12 - At 10 µM in the luminal part of the SHIME, SN-38 has no clear effect on both the luminal and the mucosal microbial composition as shown with DGGE.



Supplementary Figure 3.13 - Relative abundances of the 20 most abundant genera of the microbial community of the M-SHIME run with SN-38 based on Illumina Miseq sequencing of the 16S rRNA gene.

CHAPTER 4

Oral microbiota reduce wound healing capacity of epithelial monolayers, irrespective of the presence of 5-fluorouracil

This chapter has been redrafted after

Vanlancker, E., Vanhoecke, B., Sieprath, T., Bourgeois, J., Beterams, A., De Moerloose, B., De Vos, W.H. and Van de Wiele, T. Oral microbiota reduce wound healing capacity of epithelial monolayers, irrespective of the presence of 5-Flourouracil. *Submitted at Experimental Biology and Medicine.*

CHAPTER 4
Oral microbiota reduce wound healing capacity of epithelial monolayers, irrespective of the presence of 5-fluorouracil

Abstract

Oral mucositis is still one of the most painful side effects of chemotherapeutic treatment with a major impact on quality of life for cancer patients. A mounting body of evidence suggests a role for the oral microbiome in mucositis. However, the underlying mechanisms remain elusive. In this work, we have investigated the interactions between the host, the microbiome and chemotherapeutic treatments in more detail. To this end, the effect of 5-fluorouracil (5-FU), commonly inducing mucositis, was assessed on a co-culture model that consists of an epithelial cell layer and a biofilm derived from oral microbiota from different types of samples (saliva, buccal swabs and tongue swabs) and donors (healthy individuals and patients suffering from mucositis). After 24 h of co-incubation, all oral microbial samples were found to reduce wound healing capacity with 26 ± 15 % as compared with untreated condition. Compared with saliva and tongue samples, buccal samples were characterized by lower bacterial cell counts and hence higher wound healing capacity. For samples from healthy individuals, an inverse correlation was observed between bacterial cell counts and wound healing capacity, whereas for patients suffering from mucositis no correlation was observed. Moreover, patient-derived samples had a less diverse microbial community and higher abundances of pathogenic genera. No major impact of 5-FU on wound healing capacity or the composition of the microbiome was seen at physiologically relevant concentrations in the mouth. In conclusion, bacterial cell count is inversely correlated with wound healing capacity, which emphases the importance of oral hygiene during oral wound healing in healthy individuals. However, future research on extra measures besides oral hygiene is needed to assure a good wound healing during mucositis, as for patients the bacterial composition seems also crucial. The direct effect of 5-FU on both the microbiome and wound healing is minimal, pointing to the importance of the host and its immune system in chemotherapy-induced microbial shifts.

1. Introduction

Oral mucositis is a painful and debilitating complication of cancer treatment with a major impact on the quality of life of the patient. Its frequency is high but varies depending on the type of treatment with around 20-40 % incidence in conventional chemotherapeutic treatment of solid tumors, to almost 100 % for high-dose chemotherapy prior to HSCT or radiotherapy for head and neck cancer (Sonis 2007, Lalla et al. 2008, Villa and Sonis 2015). Although it is one of the most studied toxicities of cancer treatment, only few therapeutic agents are available for oral mucositis (Villa and Sonis 2016). One of the chemotherapeutic agents with high risk of developing mucositis is 5-FU (Villa and Sonis 2015), an antimetabolite that inhibits thymidylate synthase (TS) and is incorporated in DNA and RNA (Grem 2000, Longley et al. 2003). The incidence of developing grade 3-4 oral mucositis (i.e. confluent ulcers and unable to eat solids) in case of 5-FU treatment is more than 15 % (Sonis et al. 2004a). During continuous infusion (22 h), plasma levels of 5-FU range from 3 to 10 µM and saliva levels from 0.08 to 0.8 µM (Joulia et al. 1999, Takimoto et al. 1999). Previous research has indicated that some oral species are sensitive to 5-FU starting from 0.4 µM (Vanlancker et al. 2016).

The pathogenesis of mucositis is described by the 5-stage model of Sonis (2007). Shortly, ROS are generated in the initiation phase, followed by the activation of transcription factors, such as NF-κB. These induce the production of pro-inflammatory cytokines and activate other signaling pathways. Feedback-loops induce more inflammation and apoptosis which lead to the ulceration phase, in which bacteria colonise the ulcers and can penetrate to the submucosa. In most cases, spontaneous healing takes place within two to three weeks after completion of the treatment. Although this last phase is of great importance in terms of recovery and further continuation of the cancer treatment, it is also the least understood (Sonis 2007).

More and more evidence is emerging on the role of the oral microbiome in the pathogenesis of oral mucositis (Stringer and Logan 2015, Vanhoecke et al. 2015b, Vasconcelos et al. 2016). Microbiota can play a negative role in mucositis and induce infection of the ulcers which encourages the use of antimicrobial agents. However, no clinical guidelines have been formulated regarding the use of antimicrobial agents due to insufficient and conflicting scientific data (Saunders et al. 2013, Vanhoecke et al. 2015b). Microbiota may also be involved in phases other than the ulceration phase, and this role can be both positive and negative (van Vliet et al. 2010). Microbiota are for example able to influence the activation of TLR, NF-κB and MAPK, which are all proteins involved in important signaling pathways regulating mucositis. This way, microbiota might contribute to a higher tissue inflammation level and therefore increase apoptosis rate (Stringer and Logan 2015).

Clinical studies have shown shifts in the oral microbial profile of patients, both after chemo- and radiotherapy. However, the great variability in patient population, sample collection and technical methods to analyze the microbiota makes it difficult to generalize conclusions (Vanhoecke et al. 2015b). It seems that for blood cultures and oral swabs taken during chemotherapy, the most frequently isolated Gram-negative species are *Enterobacteriaceae* spp., *Pseudomonas* spp., and *E. coli*, whereas *Staphylococcus* spp. and *Streptococcus* spp. are the most frequently isolated Gram-positive species (Napenas et al. 2007, Vanhoecke et al. 2015b). Not only microbial composition, but also functional factors such as the mucus layer and microbial adhesion can be affected by the cancer treatment (Stringer et al. 2009c, Vanhoecke et al. 2015b). Moreover, oral microbiota may regulate wound recovery, with positive or negative effects depending on the species and the bacterial density (Edwards and Harding 2004, Laheij et al. 2013, De Ryck et al. 2015). These factors will depend on both the donor and on the specific site in the oral cavity, as they each have their own microbial community (Segata et al. 2012). For example, the saliva microbiome resembles the tongue microbiome but is distinct from the buccal mucosal microbiome (Segata et al. 2012).

In this study, we further investigated the role of oral microbiota on wound healing capacity and the effect of chemotherapy on both the microbiota and wound healing in an *in vitro* co-culture model that was previously optimized (De Ryck et al. 2014). First, the toxicity of 5-FU towards oral epithelial cells was determined using the MTT/SRB cytotoxicity tests. Next, the impact of oral microbiota and 5-FU, and the combination thereof, on epithelial wound healing were studied in the co-culture model for 24 h, with a special focus on the potential impact of the type of oral sample and donor variability.

2. Materials and methods

2.1 Cell culture

The TR146 cell line, obtained from the Laboratory of Experimental Cancer Research (Ghent University Hospital), is an oral squamous cell carcinoma cell line isolated from a local lymph node metastasis (Rupniak et al. 1985). Cells were cultured at 37°C, 10 % CO_2 and 90 % relative humidity in Dulbecco's modified Eagle's Medium (DMEM) (Gibco) with 10 % heat inactivated fetal bovine serum (Greiner Bio-one), 100 IU/mL penicillin (Gibco), 100 µg/mL streptomycin (Gibco) and 2.5 µg/mL amphotericin B (Gibco).

2.2 Oral samples

Oral samples were obtained from healthy children or patients suffering from oral mucositis (Ethical approval from Ghent University hospital, Belgian Registration number B670201112526), all aged 6-14 years. All patients were treated for hematological

malignancies. Three types of samples were collected: saliva, buccal swab and tongue swab. All samples were collected at least 2 h after eating or brushing teeth and before sampling the oral cavity of the individuals was flushed with drinking water. For the buccal and tongue samples, a sterile cotton swab was gently wiped ten times along the inner cheek or on the dorsal side of the tongue and subsequently dissolved in 1 mL of PBS.

2.3 Chemicals

A filter-sterilized stock solution of 100 mM 5-FU (Sigma Aldrich) was prepared in DMSO and further diluted to 75, 50, 20, 10, 5, 1, 0.1, 0.01 mM in DMSO. Stock solutions were further diluted (1:1000) in culture medium for the experiments.

2.4 MTT/SRB test

To test the cytotoxicity of 5-FU, an MTT/SRB test was performed. The MTT assay (Mosmann 1983) was used to measure the mitochondrial activity and the SRB assay (Vichai and Kirtikara 2006) to measure cellular protein content. TR146 cells were seeded in 96 well plates at a density of 40 000 cells/well (100 µL DMEM with serum/well). After 24 h, medium was discarded and 100 µL serum-free, antibiotic-free DMEM was added together with different 5-FU concentrations (0.01-100 µM). DMSO (1:1000) was used as a control. All plates were incubated at 37°C and 5 % CO_2. After 24 h, 48 h and 5 days an MTT and SRB test was performed. Six biological replicates were included for each 5-FU concentration and for each time point. For the MTT-assay, 20 µL MTT (3-(4,5-demethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (5 mg/mL in PBS) was added and incubated for 2 h at 37°C. All medium was removed and formazan crystals were resuspended in 100 µL DMSO. The absorbance was measured at 570 nm. For the SRB (sulforhodamine B) assay, cells were fixated by adding 25 µL 50 % trichloroacetic acid (TCA) and incubated for 1 h at 4°C. After removal of the TCA, the plate was rinsed with water and dried. Next, 75 µL SRB solution (0.4 % in 1 % glacial acetic acid) was added and the plate was incubated for 30 minutes at 4°C. The plate was then rinsed with 1 % glacial acetic acid and dried. The stained cells were resuspended in 200 µL 10 mM Tris buffer and the absorbance was measured at 490 nm.

2.5 Co-culture model

To investigate the interactions of oral microbiota and oral epithelial cells without direct contact, we used an oral *in vitro* model described by De Ryck et al. (2014) (Figure 4.1). Briefly, the model consists of a 24-well Transwell® plate with removable inserts with a polycarbonate membrane of pore size 0.4 µm (Corning Incorporated). In the apical part, 20 µL of the bacterial suspension was brought on top of a solidified agar/mucin solution (75 µL, 5 % porcin mucin

Type II, 0.8 % agar). PBS was used as a control. In the basolateral side, epithelial cells were seeded at a density of 250 000 cells/well and at confluency a wound healing assay was performed (see below). During co-culture, the inserts with the microbiota were transferred to the wells containing the epithelial cells and incubated at 37°C, 10 % CO_2 in serum-free, antibiotic-free DMEM (Gibco) with 5-FU (10 µM) or DMSO as a control (1:1000). After 24 h of co-culture, inserts were removed and 100 µL PBS was added to collect the bacteria for further analysis. For each of the seven donors (4 healthy individuals and 3 patients suffering from mucositis), a buccal sample, a saliva sample and a tongue sample as well as a blank (without microbiota) was tested in this co-culture model, each with and without 10 µM 5-FU (Figure 4.1). Each condition was tested in triplicate or quadruplicate.

2.6 Wound healing assay

During co-incubation, a wound healing assay was performed based on the protocol by De Ryck et al. (2014) (Figure 4.1). TR146 cells were stained with Vybrant DiI cell labelling solution (Life Technologies) before seeding in 24 well Transwell® plates at 250 000 cells/well. At the start of the experiment, two scratches were made in the confluent monolayer using a sterile 100 µL pipette tip. Cell medium was discarded to remove cellular debris and 1 mL of new serum-free, antibiotic-free DMEM was added to the cells. At four selected fields per well and at each time point, images of the wound were acquired using a fully automated widefield fluorescent microscope (Nikon Ti, Nikon Instruments), equipped with a 4x/0.15 Plan Achromat objective and EM-CCD camera (Andor Ixon+, Andor Instruments). The surface area of the wound was calculated for each time point using a home-written script for FIJI freeware (http://fiji.sc) that is available upon reasonable request (www.uantwerpen.be/Cell-group/scripts). In brief, the DiI counterstained time-lapse images are first pre-processed by background subtraction and local contrast enhancement, after which the non-damaged part of the cell monolayer is detected by a combination of variance, maximum and Gaussian blur filtering, and segmented using a user-defined or automatic threshold. The inverse of this mask is selected as wounded area. The relative wound size was calculated by normalizing to the wound area at 0 h.

At the end of the wound healing experiment, metabolic activity and viability of the epithelial cells was evaluated with an MTT-assay. To each well, 1 mL of serum-free, antibiotic-free DMEM and 200 µL MTT (5 mg/mL in PBS) was added and incubated for 2 h at 37°C. After removal of the medium, the formazan crystals were dissolved in 1 mL DMSO. Absorbance at was measured at 540 nm (200 µL) (Infinite F50 Tecan). Percentage of viability, compared to the control, was calculated.

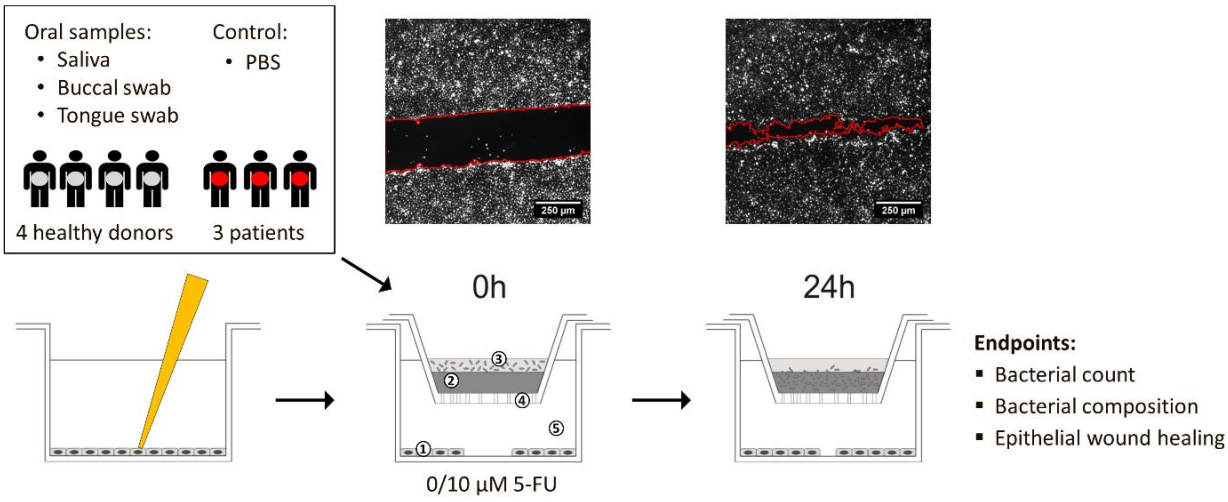


Figure 4.1 - Experimental set-up of the co-culture model with 1) oral epithelial TR146 cells stained with DiI, 2) agar/mucin layer, 3) microbial biofilm or PBS as a control, 4) polycarbonate membrane with 0.4 µm pores, 5) DMEM with 0 µM or 10 µM 5-FU. Fluorescent images show examples of wounds (red line) at 0 h and 24 h (based on De Ryck et al. (2014)).

2.7 Colony-forming units (CFU)

To measure the number of viable cells present in the insert, the oral samples (saliva, oral swab, tongue swab) were plated using Brain Heart Infusion (BHI)-agar plates. A dilution series was made and 10 µL of bacterial suspension was plated in triplicate.

2.8 Flow cytometry

The number of intact and damaged bacterial cells in the insert after 24 h was measured by flow cytometry as described in Chapter 2, section 2.4 based on Van Nevel et al. (2013).

2.9 Microbial community analysis

Total DNA was extracted from the pellet of the bacterial suspension on the filter at t = 24 h or the pellet of 100 µL of the sample at t = 0 h as described in Chapter 3, section 2.4. On all samples, DGGE was performed using the 338F-GC and 518R primers targeting the V3 region of the 16S rRNA gene as described in Chapter 3, section 2.5.1. Richness and Gini coefficient were calculated based on the DGGE profiles. The number of bands that were detected is a measure for richness. The Gini coefficient, based on the Pareto-Lorenz curves, is a measure for evenness of the community (Marzorati et al. 2008). Higher Gini coefficients represent a lower evenness, indicating the dominance of a small fraction of species in the community. Illumina sequencing of the V3-V4 region of the 16S rRNA gene was performed on one replicate of each condition for the saliva samples of all individuals by LGC Genomics (Berlin, Germany) on the MiSeq platform as described in Chapter 3, section 2.5.2. The Illumina

sequencing data were deposited to the European Nucleotide Archive (SRA) with study number PRJEB20819.

2.10 Statistical analysis

All statistical analyses were performed in R (version 3.3.2). Mixed-model regression of MTT and SRB data was performed for each time point with the concentration as categorical predictor. A random intercept effect was incorporated for each replicate measurement. In order to make correct statistical inference, all models were evaluated for normal distributed residuals with homogenous variance, by Shapiro Wilk tests ($p>0.05$) and visually by Q-Q plots. Model parameters were estimated by maximum likelihood. When a significant concentration effect was present (ANOVA, $p < 0.01$), the categories were compared pair-wise by posthoc analysis using Tukey's Honestly Significant Difference (HSD) method. All tested concentrations were compared with the control condition (0 µM) and differences were considered significant at $p<0.05$.

For all other basic statistics, linear models were build using forward selection of parameters (fixed factors and interactions) on the scaled and centered data. All models were evaluated for normal distributed residuals with homogenous variance. When a significant effect was present, the posthoc analysis was performed using multiple comparisons with Benjamini Hochberg correction. When interactions were present, data were split in subgroups to define significant differences. Differences were considered significant at $p<0.05$.

The packages phyloseq (McMurdie and Holmes 2013) and vegan (Oksanen 2016) were used for microbial community analysis. Heatmaps were generated with the pheatmap package and order-based Hill's numbers (Hill 1973) were calculated. NMDS plots of the bacterial community data were created based on the Bray-Curtis distance measures. Significant differences were identified by means of Permutational ANOVA (PERMANOVA) using the *adonis* function (vegan).

3. Results

3.1 5-FU toxicity to oral epithelial TR146 cells

To assess the direct toxicity of 5-FU towards TR146 oral epithelial cells, an MTT/SRB test was performed after 24 h, 48 h and 5 days of treatment (Figure 4.2). The SRB test showed a significant decrease ($p<0.05$) in protein content starting from 10 µM for all time points. These decreases ranged from a drop with 10 % for 10 µM after 24 h to 63.6 % for 100 µM after 5 days. The MTT test showed a small but significant ($p<0.05$) increase in mitochondrial activity for some time points at low concentrations of 5-FU (0.01-1 µM). At higher concentrations (starting from 10 µM), small decreases were observed after 24 h and 48 h. Viability dropped

to less than 50 % after 5 days of treatment with 5-FU at levels higher than 20 µM ($p<0.05$). Together these data show that 5-FU was toxic for TR146 cells starting from 20 µM after 24 h and starting from 10 µM after 48 h or 5 days.

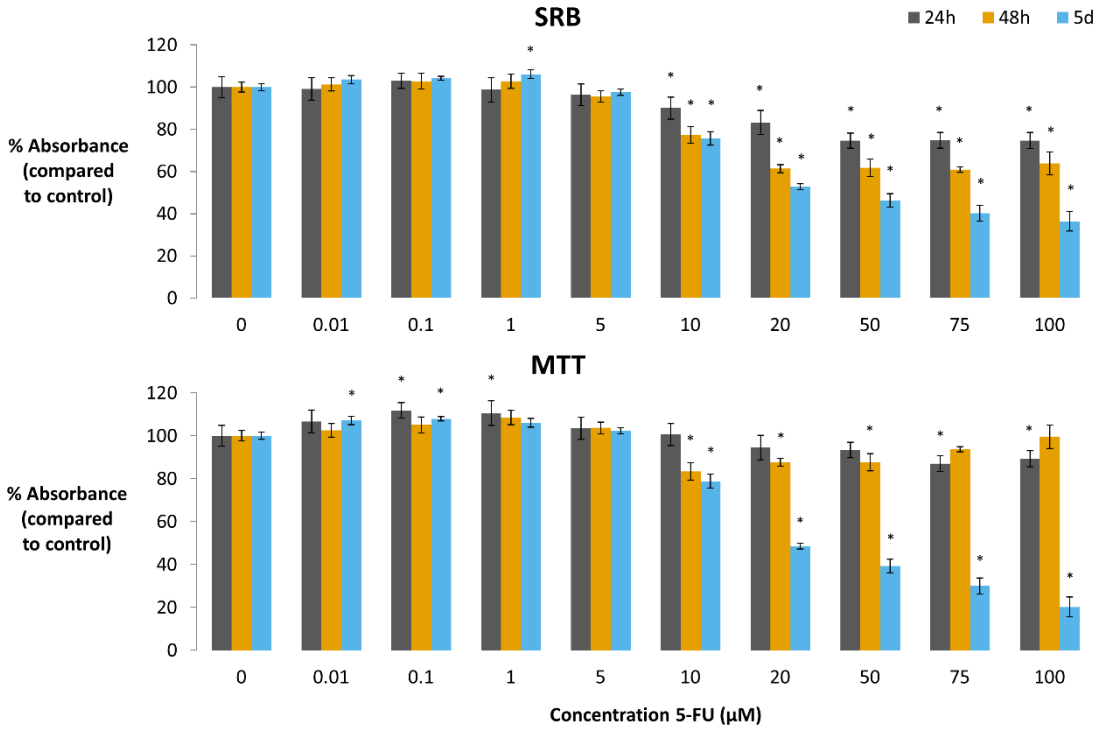


Figure 4.2 - MTT and SRB toxicity test of 5-FU (0.01-100 µM) on oral epithelial TR146 cells (AV±SD, n=6). Significant deviations from the control condition (0 µM) are indicated by the asterisks ($p<0.05$).

3.2 Bacterial cell counts are determined by sample and donor type

For seven donors (4 healthy individuals and 3 patients suffering from mucositis), three types of samples (saliva, buccal swabs and tongue swabs) were investigated in a co-culture model (Figure 4.1). In this model, the microbial sample was incubated for 24 h on an agar/mucin layer in indirect contact with oral epithelial cells. Each sample was tested in absence of presence of 10 µM 5-FU in the basolateral compartment. Both the initial (directly after taking the sample, t = 0 h) and final (after 24 h of co-culture, t = 24 h) bacterial cell counts were evaluated (Figure 4.3A). Depending on the type of sample and the type of donor, the initial bacterial concentration ranged between 1 and 5 log CFU. With regards to the different oral sample types, a clear distinction was observed between buccal swabs on the one hand and saliva and tongue swabs on the other. The initial (t = 0 h) bacterial concentration in buccal swabs (1.9 ± 1.3 log CFU) was significantly lower compared to saliva (3.7 ± 1.8 log CFU, $p<0.001$) and tongue swabs (3.6 ± 1.0 log CFU, $p<0.001$). Despite this variation in initial number, all samples were able to grow up to a concentration of 7-8 log CFU after 24 h of

co-culture in the *in vitro* model. The difference in concentration, depending of the sample type, was still present after 24 h, with slightly lower bacterial cell counts for the buccal swab amended wells (7.4 ± 0.4 log cells) compared to saliva (7.9 ± 0.4 log cells, $p<0.001$) and tongue swab amended wells (7.8 ± 0.4 log cells, $p<0.001$). Also the type of donor affected the bacterial cell counts. While patient samples displayed a 2-3 log lower initial bacterial concentration compared to healthy individuals ($p<0.001$), no significant differences were noted after 24 h in the co-culture model ($p=0.12$). Surprisingly, treatment with 5-FU did not alter bacterial cell counts at 24 h ($p=0.60$). Thus, bacterial cell counts are determined by both sample type and donor type, but are not affected by 5-FU.

3.3 Buccal-derived samples have lower microbial diversity, compared to saliva and tongue amended samples

DGGE analysis (Figure 4.3B and Supplementary Figure 4.1) showed that differences in microbial diversity between sample types was dependent on the type of donor, as an significant interaction between donor type and sample type was seen. For healthy individuals, the microbial community of the buccal swab amended wells was lower in richness and evenness, compared to saliva (for both $p<0.001$) and tongue swab amended wells (for both $p<0.001$) at 24 h. For patients, only a significant increase in Gini coefficient was seen for buccal amended wells, compared to saliva ($p=0.0055$). For each donor, Bray-Curtis analysis of DGGE profiles also showed significant differences between the different sample types (Supplementary Table 4.1).

3.4 Patient-derived samples are less diverse and enriched in pathogenic genera as compared to healthy donor samples

With regards to donor type, DGGE analysis showed different responses depending on the type of sample. For saliva and tongue swab amended wells, a lower richness ($p<0.001$ for both) and evenness ($p=0.0084$ and $p<0.001$) was observed for patient-derived samples at 24 h, compared to wells with samples from healthy individuals (Figure 4.3B). However, no differences were seen for buccal swab amended wells (richness $p=0.91$ and evenness $p=0.77$). The high cell density in saliva samples allowed for performing Illumina sequencing (guaranteeing high-quality data acquisition). As could be expected, the results showed clear differences between donor types (Figure 4.4 and Supplementary Figure 4.2). In correspondence with DGGE results, diversity parameters based on amplicon sequencing were lower for patient-derived samples, compared to samples from healthy individuals (Hill number order 0, $p=0.0067$; order 1, $p=0.028$; order 2, $p=0.026$) (Figure 4.4C). Bray-Curtis analysis at OTU level revealed that 16.0 % of the variation in the composition of the saliva samples could

be attributed to the type of donor (p=0.0016). Visualization by NMDS plots confirmed the major impact of donor type as all patient-derived samples cluster to one side of the plot (Figure 4.4B). At 24 h, patient-derived saliva samples were more dominated by Lactobacillales (containing *Streptococcus*, *Abiotrophia* and *Enterococcus*) (95.3 ± 6.9 %) compared to samples derived from healthy individuals (45.3 ± 23.0 %) (p<0.001). In contrast, *Veillonella* is more abundant in samples derived from healthy individuals at 24 h (50.8 ± 24.3 %) in comparison with patient-derived samples (3.8 ±6.4) (p<0.0023). The initial (t = 0 h) samples from patients contained also more pathogenic genera, for example 25.5 % of *Porphyromonas* for patient 1, 2.8 % of *Enterococcus* and 3.3 % of *Staphylococcus* for patient 2 and 40.8 % of *Porphyromonas* and 12.7 % of *Mycoplasma* for patient 3. These genera were not (*Enterococcus* and *Mycoplasma*) or at much lower abundances (*Staphylococcus* 0-0.04 %, *Porphyromonas* 0.2-2.3 %) detected in the samples derived from healthy individuals. Interestingly, the initial microbial composition of the saliva samples of healthy individuals 1 and 2 on the one hand, and 3 and 4 on the other hand were very similar. This can be explained by the fact that these were samples from siblings, living in the same environment and having similar eating habits. In brief, patient samples had lower microbial diversity and higher abundance of pathogenic genera.

3.5 5-FU have no major impact on bacterial composition

DGGE showed that 5-FU did not affect richness (p=0.83) nor evenness (p=0.069) of the bacteria. Bray-Curtis analysis showed that only for patient 2, a significant effect of 5-FU on the microbial profile could be detected based on the DGGE profile (p=0.0014) (Supplementary Table 4.1). For all sample types of this patient, two dominant bands clearly disappeared following 5-FU treatment (Supplementary Figure 4.1). Similar to DGGE, Illumina sequencing showed that 5-FU treatment did not significantly affect the bacterial diversity (Figure 4.4C). However, following 5-FU treatment, a general trend in increased *Streptococcus* abundance (from 40.6 ± 26.7 % to 68.1 ± 25.5 % ; p=0.099) and of decreased *Veillonella* abundance (from 44.7 ± 34.8 % to 26.8 ± 23.4 % ; p=0.32) was observed (Figure 4.4A). In contrast to the other individuals, wells derived from patient 3 were dominated by *Abiotrophia* after 24 h of co-culture both with and without 5-FU. More specifically, *Prevotella* abundance increased following 5-FU treatment for samples derived from healthy individual 3 and 4 (0.4 % to 3.8 % and 0.4 % to 2.2 % respectively). For patient 2, *Enterococcus* and *Streptococcus* were the most abundant genera in the untreated wells (70.4 % and 29.2 % respectively), whereas in presence of 5-FU *Streptococcus* dominated with 98.9 %. This result confirmed the changed DGGE profiles of patient 2 following 5-FU treatment (Supplementary Figure 4.1). Altogether, these results indicate small yet non-significant changes in the composition of the biofilm following 5-FU treatment (based on Bray-Curtis dissimilarities on OTU level, p=0.66).

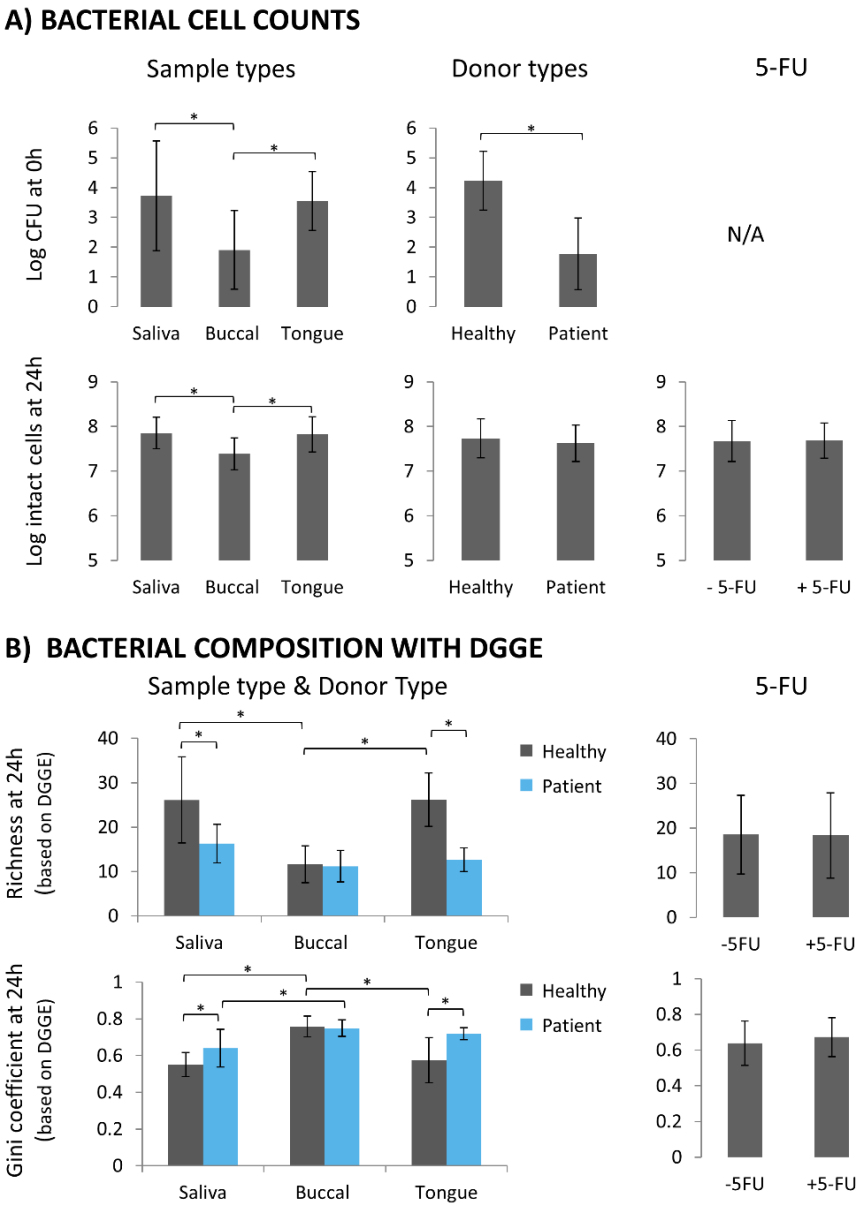


Figure 4.3 - Bacterial cell counts and composition of microbiota derived from different sample and donor types cultured in the oral co-culture model in presence or absence of 5-FU. A) Bacterial cell counts at t = 0 h and t = 24 h (AV±SD); B) Richness and Gini coefficient as measure for bacterial diversity by DGGE (AV±SD). Significant differences between groups are indicated by the asterisks (p<0.05).

3.6 Bacterial composition changes after 24 h of co-culture

Finally, a significant change in bacterial composition was observed with Illumina sequencing attributed to sampling time points (t = 0 h vs. t = 24 h) (p=0.01), which explained 13.3 % of the variation in all samples (based on Bray-Curtis dissimilarities on OTU level). This difference was also visible in the NMDS plot (Figure 4.4B). Moreover, all Hill numbers showed a decrease in diversity at 24 h compared to the initial samples (Hill number order 0, p<0.001; order 1, p<0.001; order 2, p<0.001) (Figure 4.4C). *Streptococcus* and *Veillonella* were the

dominating genera in the saliva samples after 24 h in the *in vitro* model (together 95.6 ± 4.2 %), apart from the control sample derived from patient 2, which was dominated by *Enterococcus* and the samples derived from patient 3, which were dominated by *Abiotrophia* (Figure 4.4A). Next to *Streptococcus* and *Veillonella*, the initial saliva samples were also populated by *Prevotella*, *Neisseria*, *Granulicatella*, *Haemophilus*, *Actinomyces*, *Porphyromonas*, *Fusobacterium*, and *Megasphaera*, of which levels depended on the donor. A lot of this diversity was lost during the 24 h incubation in the co-culture model. For some donors, most genera were still present albeit at relatively low abundances.

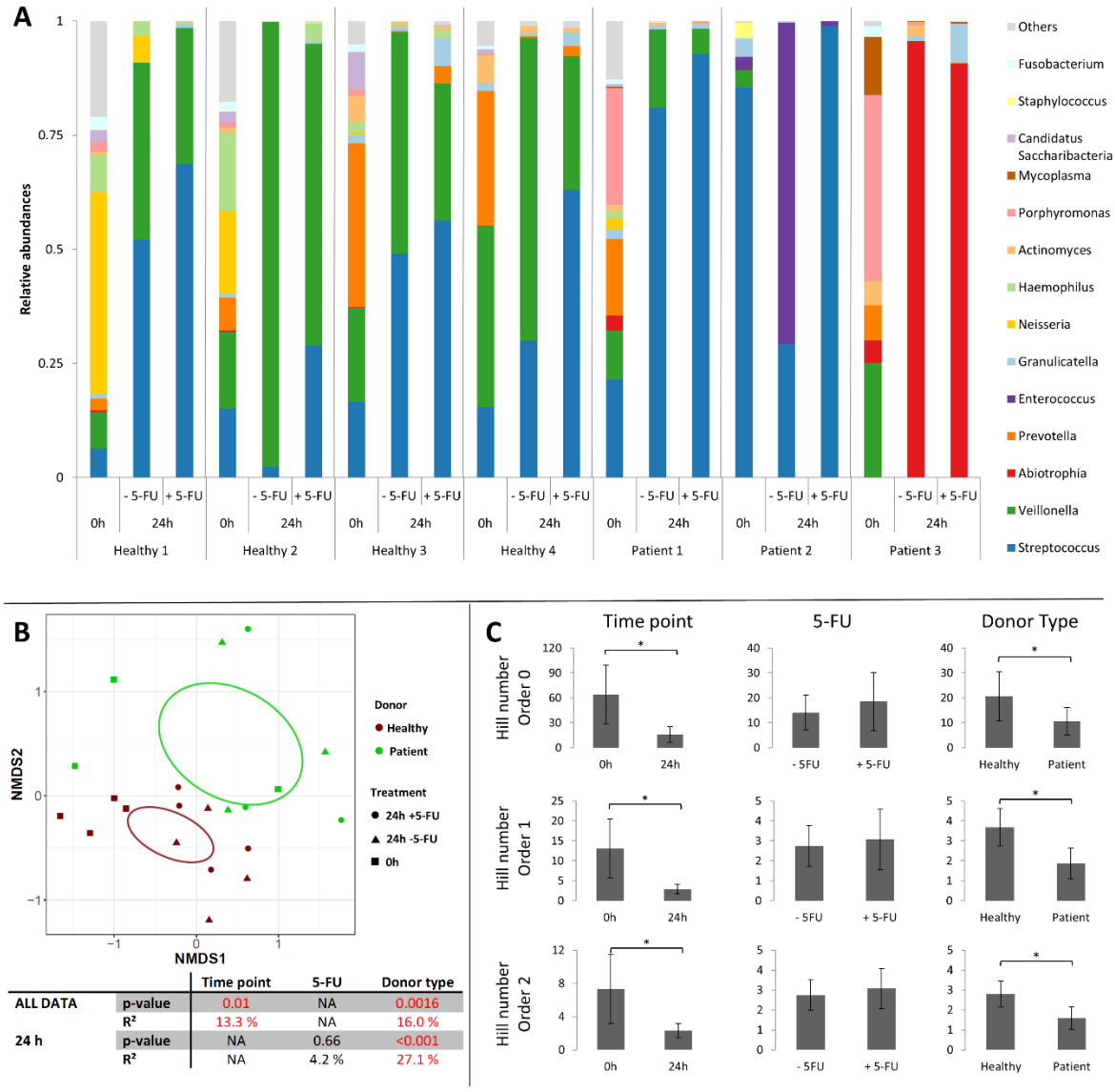


Figure 4.4 - Illumina sequencing of the 16S rRNA gene of the microbiota in the saliva samples. A) Barplot representing the 14 most abundant genera; B) NMDS plot with 95 % confidence ellipsoids of the mean, p-values and R^2 for different confounding factors based on Bray-Curtis dissimilarities; C) Hill numbers order 0, 1 and 2 representing richness, evenness and diversity respectively (AV±SD). Significant differences between groups are indicated by the asterisks (p<0.05).

3.7 Epithelial wound healing is reduced by oral microbiota, irrespective of the presence of 5-FU

To investigate the closure of artificially induced wounds in an epithelial monolayer over time, we followed wound healing in a separate set-up with and without microbiota derived from a buccal swab from a healthy individual in presence or absence of 5-FU (Figure 4.1). Compared to the (unchallenged) control wells, epithelial cell wound healing slowed down in presence of microbiota starting from 16 h, eventually resulting in a 16 % lower wound healing after 25 h (Figure 4.5A). The presence of 5-FU had no effect on the wound healing capacity and this was independent of microbial presence.

This experiment showed that co-culture with microbiota reduces wound healing of oral epithelial cells. This effect might, however, be caused by different bacterial cell counts and composition, which have been shown to depend on the type of donor and the sample type. Indeed, although a general reduction (25.9 ± 15.1 %) of wound healing capacity was observed by the addition of oral microbiota, differences could be noticed between the sample and donor types (Figure 4.5).

First, addition of microbiota derived from saliva and tongue swabs had a more detrimental effect on wound healing in comparison with buccal-derived microbiota ($p=0.0051$ for saliva; $p=0.041$ for tongue) (Figure 4.5B). Regarding the type of donor, no difference in wound healing capacity was noticed ($p=0.95$). However, plotting the wound opening at 24 h as a function of the bacterial cell counts revealed two different trends between healthy and patient samples (Figure 4.5C). Microbial samples from healthy individuals displayed a linear relationship with each additional log CFU of bacterial cells resulting in a 15.2 % increase in wound opening ($p=0.00082$). Independent of microbiome composition, this is indicative (adjusted $R^2 = 0.17$) of a higher wound healing capacity at lower bacterial loads. However, no such trend could be observed for patient samples ($p=0.13$). Again, no modulating effect of 5-FU on wound healing was observed after 24 h in the presence ($p=0.49$) and absence ($p=0.21$) of microbiota. An MTT assay performed after 24 h of co-culture showed no effect of sample type ($p=0.26$) or type of donor ($p=0.23$) on the cell viability of TR146 cells. A small but significant increase in epithelial cell viability was observed following 5-FU treatment in presence of microbiota (89.5 ± 11.0 % to 95.3 ± 11.4 %, $p=0.004$), whereas no effect was observed in absence of microbiota ($p=0.94$). Together these data indicate that wound healing potential is determined by both bacterial cell count and bacterial composition.

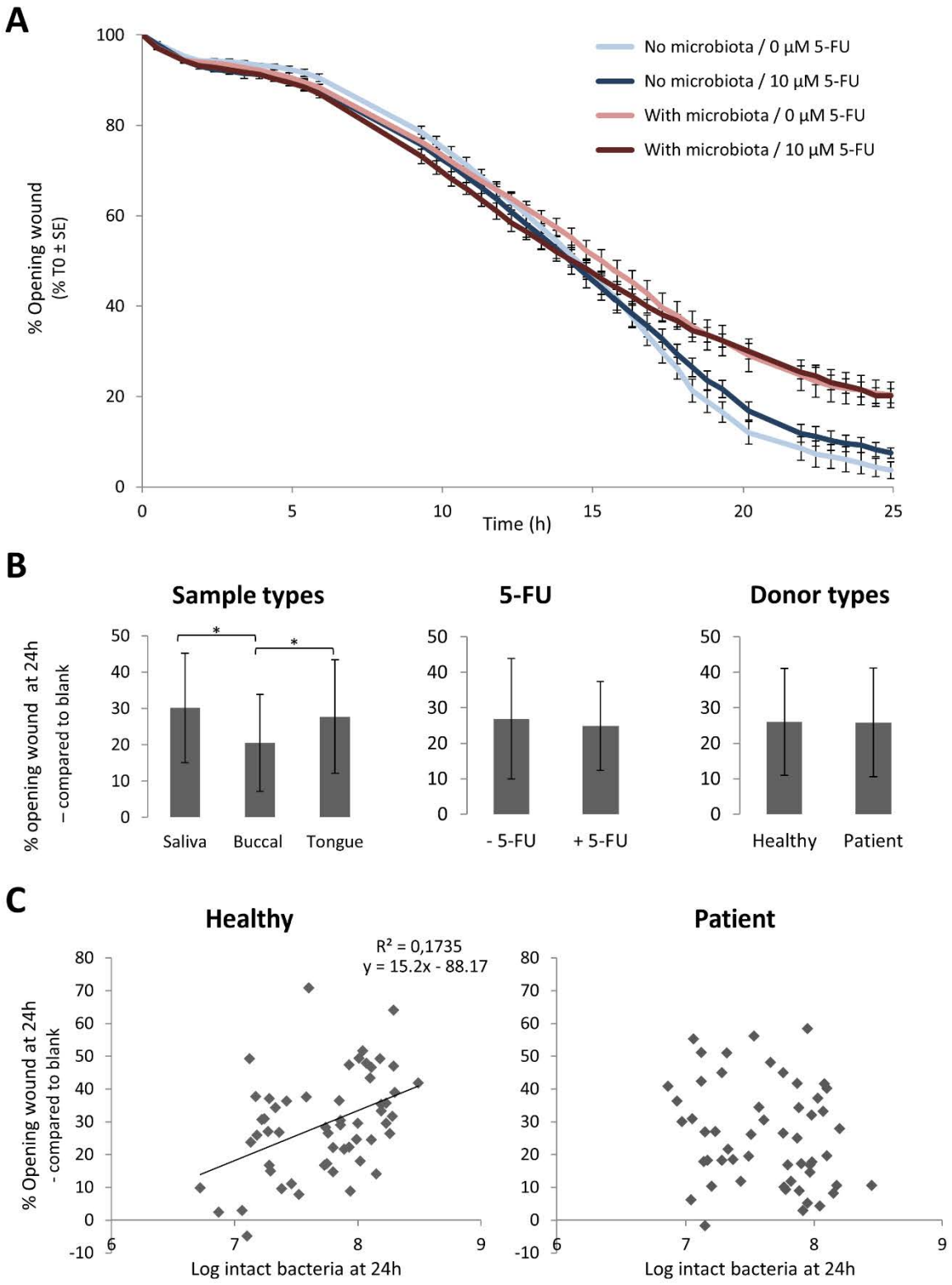


Figure 4.5 - A) Oral microbiota derived from a buccal swab reduce wound healing capacity of oral epithelial cells in an *in vitro* mucosa model, irrespective of the treatment with 5-FU (10 µM) (AV±SE); B) Sample type affect wound healing capacity, whereas donor type and presence of 5-FU do not (AV±SD); C) For healthy individuals, a positive correlation between the opening of the wound and bacterial cell count at 24 h was observed, but for patients no such trend was noticed.

4. Discussion

Oral mucositis is a debilitating side effect of chemotherapeutic treatment in which microbiota are more and more shown to play an important role. In this study, we investigated the interactions between the oral microbiome, oral epithelial cells and a chemotherapeutic (5-FU) using an *in vitro* co-culture model. As wound healing is crucial in recovering from mucositis, this was one of the functional endpoints in the model apart from microbial numbers and composition.

Our data showed that oral microbiota reduced wound healing capacity for all seven donors with 25.9 ± 15.1 %. Previous research using the same *in vitro* model, showed that oral microbiota had similar negative effects on wound healing (De Ryck et al. 2014). However, this reduction appeared to be species- and concentration-dependent (Edwards and Harding 2004, De Ryck et al. 2015). Not only in the oral cavity, but also in other body sites wound healing capacity can be concentration dependent. In chronic skin wounds, low amounts of microbiota can improve wound healing, whereas in infectious conditions with high bacterial loads wound healing capacity is significantly reduced (Edwards and Harding 2004). Our data for healthy individuals confirmed previous research, as lower bacterial cell counts correlated with higher wound healing capacity. This encourages the use of good oral hygiene during mucositis, shown previously to be of high importance in oral mucositis, as colonization of the ulcers by microbiota may prolong the healing phase (Keefe et al. 2007, Villa and Sonis 2016). However, for patients that are in the acute mucositis phase, more measures might be needed, as we have shown that for such patients wound healing capacity was independent of the bacterial cell counts. This indicates that also bacterial composition might be important in acute mucositis patients. De Ryck et al. (2015) indeed showed that wound healing capacity seems to be species-dependent with *Klebsiella oxytoca* having a deleterious effect on wound healing, whereas *Streptococcus mitis* and *S. oralis* stimulated wound healing.

Further, we observed differences in the composition and diversity of oral microbiota derived from patients suffering from mucositis compared to healthy individuals. The abundance of *Lactobacillales* was higher in patient samples in comparison with healthy individuals and the diversity of samples derived from patients was lower. Our results are in accordance with a prospective study with 454-sequencing of mucosal samples also showing a lower diversity in patient samples compared to reference individuals (Ye et al. 2013). Moreover, the Illumina data from our study revealed the presence of larger numbers of genera containing pathogenic species, like *Porphyromonas*, *Enterococcus* and *Staphylococcus*, in the patient-derived samples, which could lead to a higher infection risk. *Porphyromonas gingivalis* was shown previously to be predictive for the development of oral ulcerations in HSCT patients (Laheij et al. 2012).

Different sites in the oral cavity are colonized with distinct microbial communities (Segata et al. 2012). In our study, buccal swabs had lower bacterial cell counts, compared to saliva and tongue swabs, leading to a higher wound healing capacity, which is in line with the previous results. DGGE also indicated lower richness and evenness in the buccal samples. This lower diversity of buccal microbiome compared to saliva and tongue samples has already been explained by extensive data derived from The Human Microbiome Project by the dominance of *Streptococcus* in buccal samples (Segata et al. 2012).

We also investigated the effect of 5-FU on different endpoints in the co-culture model. We chose to work with a dose of 10 µM, as this was the highest non-toxic concentration for TR146 cells after 24 h. Similar toxicity profiles have been recorded for other cell lines such as for Caco-2 cells (Fang et al. 2014). *In vivo* concentrations range from 3 to 10 µM in plasma and 0.08-0.8 µM in saliva following continuous treatment (Joulia et al. 1999, Takimoto et al. 1999), but significantly increase in case of DPD deficiency (Saif et al. 2009). Previous research showed a variable sensitivity among oral species towards 5-FU (Vanlancker et al. 2016). However, in our system which comprises a plethora of oral species cultured in a biofilm, we did not see an impact of 5-FU on both bacterial cell counts or wound healing. Further, 5-FU had only a minor impact on bacterial composition with an increasing trend in *Streptococcus* and a decreasing trend in *Veillonella*. *Streptococcus oralis, S. mitis* and S. *salivarius* have been shown in a previous study to be resistant to 5-FU at 10 µM, (Vanlancker et al. 2016) which might explain their ability to increase in abundance. No data are available on the sensitivity of 5-FU to *Veillonella*, however our data suggest that *Veillonella* is sensitive towards 5-FU. The results for patient 2 indicated high sensitivity of *Enterococcus* towards 5-FU, confirming previous research (Stringer et al. 2009c). Moreover, patient 2 was the only donor for which a significant effect of 5-FU on the microbial composition was shown. This indicates a donor-specific effect of 5-FU which encourages the use of a personalized approach.

At 24 h, the biofilm formed in the model was mainly dominated by *Streptococcus* and *Veillonella* for the saliva samples. Although this indicates a loss of diversity of the original saliva sample when cultured in the *in vitro* model, this loss might be due to biofilm formation. *In vivo* growth of an oral biofilm on enamel-dentin slabs in the mouth of healthy volunteers also showed a dominance of *Streptococcus* (62 %) and *Veillonella* (27 %) after 48 h (Klug et al. 2016). Although we used saliva samples, the high abundance of *Streptococcus* is more similar to buccal samples (Segata et al. 2012). We hypothesize that the use of an agar/mucin layer as a substrate promotes biofilm formation of a buccal community, despite the use of a saliva sample as a microbial source. This immature biofilm is formed by streptococci, known to be initial colonizers of the oral biofilm (Kolenbrander et al. 2010). With respect to *Veillonella*, dependency on the lactic acid produced by streptococci has been shown (Kolenbrander 2000) and therefore these species are likely to co-occur.

In conclusion, oral microbiota reduce wound healing capacity of epithelial cells with higher bacterial cell counts linked to lower wound healing capacity in healthy individuals. However, for patients suffering from mucositis the mechanism of wound healing is more related to microbial composition, rather than microbial load as their oral samples are characterized by a disturbed microbial community with higher abundances of pathogenic genera. More research on the link between oral microbial composition and wound healing capacity is needed to fully understand their role in the wound healing process in patients suffering from mucositis.

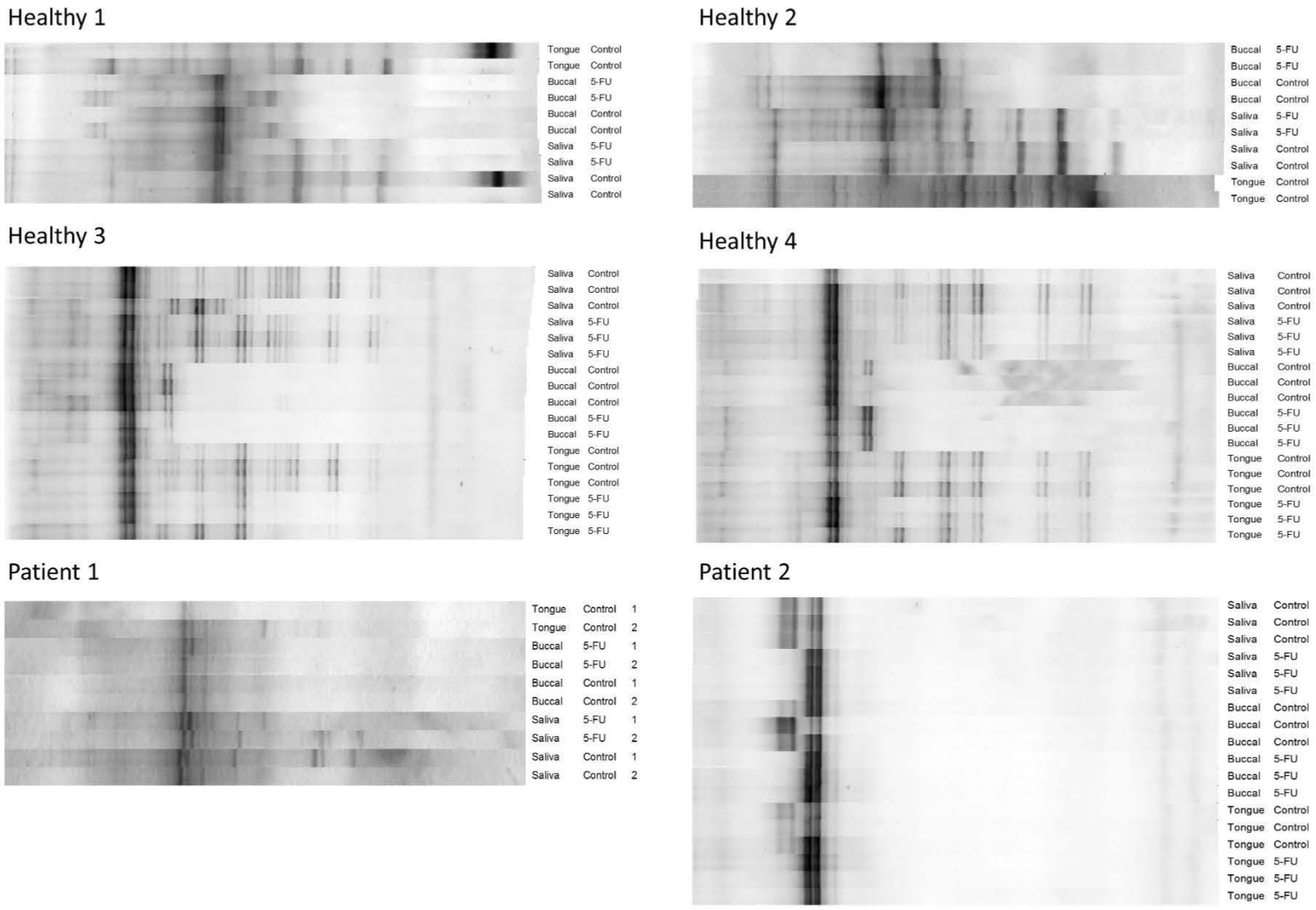
5. Acknowledgements

This work was supported by the Bijzonder Onderzoeksfonds (Ghent University grant numbers BOF13/DOC/280, BOF17/GOA/032 and BOF/11267/09; University of Antwerp grant number TTBOF29267); and the Seventh Framework Programme (FP7/2011) (grant number 299169). The authors would like to thank Charlotte Grootaert for the use of cell culture equipment for part of the work and Charlotte De Rudder and Massimo Marzorati for their review of the manuscript.

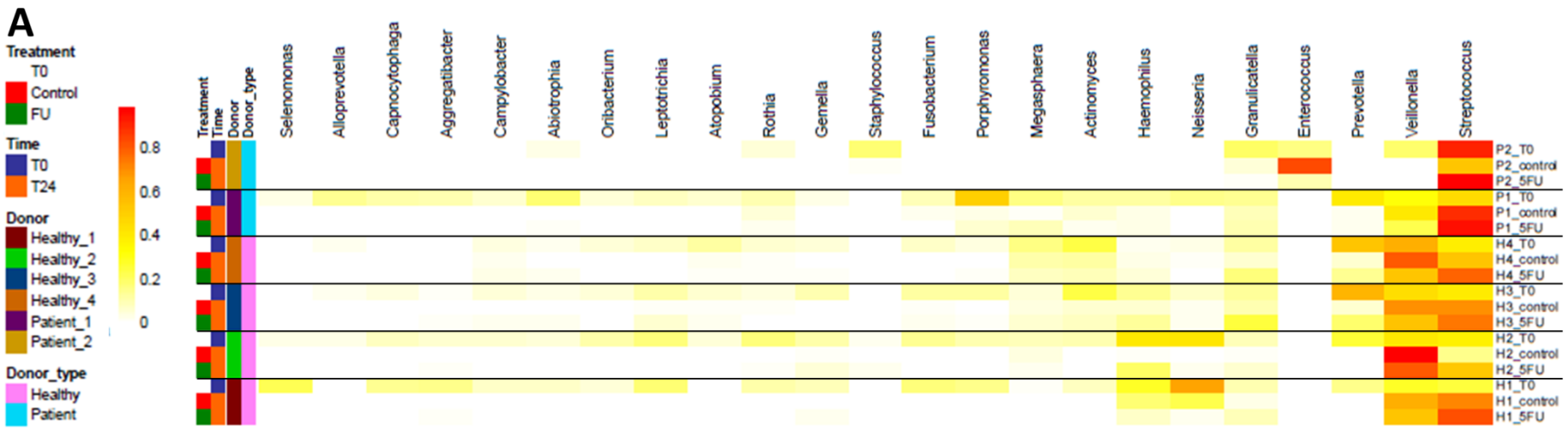
6. Supplementary information

Supplementary Table 4.1 - p-values and R^2 for different confounding factors based on Bray-Curtis dissimilarities of the DGGE data (significant values are indicated in italic)

	5-FU		Sample types	
	p-value	**R^2**	**p-value**	**R^2**
Healthy 1	0.42	11.1 %	*0.0009*	*58.3 %*
Healthy 2	0.38	12.1 %	*0.0012*	*80.5 %*
Healthy 3	0.24	8.5 %	*0.0001*	*59.3 %*
Healthy 4	0.50	4.6 %	*0.0001*	*57.7 %*
Patient 1	0.72	7.7 %	*0.0013*	*48.0 %*
Patient 2	*0.0014*	*37.7 %*	*0.0082*	*37.3 %*



Supplementary Figure 4.1 – DGGE profiles of oral samples from healthy individuals and patients, in absence and presence of 5-FU at 24h .



Supplementary Figure 4.2 - Heatmap representing the square root transformed relative abundance of genera present for at least 0.1 % based on Illumina sequencing of the 16S rRNA gene of saliva samples.

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CHAPTER 5

Longitudinal analysis of oral microbiota during chemotherapy-induced oral mucositis in pediatric patients with hematological malignancies

This chapter has been redrafted after

Vanlancker, E., De Vrieze, J., Vanhoecke, B., Desmaele, L., De Moerloose B. and Van de Wiele, T. Longitudinal analysis of oral microbiota during chemotherapy-induced oral mucositis in pediatric patients with hematological malignancies. *Manuscript in preparation*.

CHAPTER 5
Longitudinal analysis of oral microbiota during chemotherapy-induced mucositis in pediatric patients with hematological diseases

Abstract

The oral microbiome of cancer patients is subjected to several modulatory factors throughout cancer therapy. To what extent modulation of the oral microbiota contributes to the etiology or severity of oral mucositis is thus far not known. To this end, we performed a longitudinal study of the oral microbiota from five pediatric patients, treated with chemotherapy for hematological malignancies and suffering from oral mucositis. Saliva, buccal and mucositis lesions were sampled before and during chemotherapy over a 2-2.5 month period and after therapy as follow-up. Microbial community composition was determined with 16S rRNA gene based Illumina sequencing and correlations with clinical data were made. The oral microbial community displayed large dynamics throughout therapy in all patients, with patient-specific shifts. While *Streptococcus* was the predominant genus for 3 out of 5 patients, the other 2 patients showed a large variability in dominant genera over time, indicating a highly unbalanced microbiome. While the overall community composition seemed to return to its initial composition at least 1 month after therapy, a sustained impact towards lower diversity values was noted. Surprisingly, chemotherapy and mucositis had only a minor effect on microbial community composition, whereas one of the major confounding factors of our study was the use of systemic antibiotics as it majorly affected both microbial composition and diversity. Other confounding factors were sample type and sampling period, but also the use of antibacterial mouth rinse with chlorhexidine, neutropenia and inflammation. Mucositis lesions were highly dominated by streptococci, but also by more pathogenic genera as *Aggregatibacter*, *Enterococcus* and *Fusobacterium.* In conclusion, chemotherapy, antibiotic treatment and/or antibacterial mouth rinse will lower microbial diversity and create dysbiosis, which may increase the risk of infection and bacterial sepsis in neutropenic patients.

1. Introduction

Oral mucositis is one of the multiple disorders from which development and severity is suspected to be influenced by microbiota (Stringer and Logan 2015, Vanhoecke et al. 2015b, Vasconcelos et al. 2016). The exact role of the oral microbiota in mucositis is however still unclear and multiple factors can impact the oral microbial community of cancer patients during and after chemotherapy.

Shifts in the oral microbial community of cancer patients following chemotherapy have been shown in multiple studies (Napenas et al. 2007, Napenas et al. 2010, Ye et al. 2013), but no clear pattern could be observed so far due to the large variability among multiple studies in patient populations, sample types, and sample collection and analysis methods (Vanhoecke and Stringer 2015). Moreover, only a couple of studies compared the composition of the microbiota before and after chemotherapy, making it difficult to attribute microbiome shifts to chemotherapy or other disease- or therapy-associated factors (Vanhoecke et al. 2015b). The advent of next generation sequencing has however resulted in a more profound insight in how cancer therapy affects the human microbiome. The use of 16S rRNA gene clone libraries of oral buccal microbiota of 9 breast cancer patients before and after chemotherapy revealed species that had never been identified in patients before (Napenas et al. 2010). In pediatric cancer patients, 454-sequencing of oral mucosal bacterial samples showed a lower diversity and higher inter-individual variability for patients compared to healthy children. Furthermore, they found a higher microbial diversity before the start of chemotherapy and more pronounced shifts of the bacterial community by chemotherapy in patients who later developed oral mucositis (Ye et al. 2013). A recent study observed a large microbial variability over time in hospitalized cancer patients treated with chemotherapy (Galloway-Pena et al. 2017). Therefore, the importance of longitudinal follow-up to fully unravel the host-microbe interactions in oral mucositis during cancer treatment is emerging (Vasconcelos et al. 2016, Galloway-Pena et al. 2017).

Despite the variability in microbial shifts following chemotherapy, different microorganisms have been linked with mucositis or oral ulcerations. A study with immune-compromised patients showed a correlation between oral lesions and the presence of *Staphylococcus* spp., *Enterococcus* spp., and *Candida* spp. (Olczak-Kowalczyk et al. 2012). In patients undergoing HSCT, *Porphyromonas gingivalis* in particular, but also *Parvimonas micra*, *Treponema denticola*, *Fusobacterium nucleatum*, and *Candida* spp. were associated with oral ulcerations (Laheij et al. 2012). Also viruses such as *Herpes simplex virus* (HSV)-1 have been shown to be linked with the severity of mucositis (de Mendonca et al. 2012, de Mendonca et al. 2015). Microorganisms colonizing this ulcers might easily penetrate into the submucosa and cause bacteremia and sepsis (Sonis 2007). In the past, Gram-negative

bacteria were the main cause of invasive infections in neutropenic cancer patients. However, a shift towards Gram-positive infections has been shown since the 1990s (Zinner 1999) (Panghal et al. 2012). This can be explained by the prophylactic use of fluoroquinolones, the use of high-dose chemotherapy causing oral mucosal barrier disruption and the use of central venous lines increasing the risk of infections with skin bacteria, which are usually Gram-positive (Zinner 1999, Panghal et al. 2012). Viridans streptococci are an important group of Gram-positive pathogens causing infections (Shenep 2000, Tunkel and Sepkowitz 2002) and increased abundance of *Streptococcus oralis* group of viridians streptococci has been shown in saliva samples following chemotherapy and total body irradiation for allogeneic bone marrow transplantation in pediatric patients (Lucas et al. 1997).

In this study, we investigated the oral microbial composition of 5 pediatric patients treated with chemotherapy for hematological malignancies, as they highly suffer from oral mucositis. The first aim was to perform a longitudinal analysis of the oral microbial community by monitoring saliva, buccal and lesion microbiota once or twice a week for a 2-2.5 month period with 16S rRNA gene-based Illumina sequencing. The second aim was to link the oral microbial changes during this long-term period with potential confounding factors. Factors of interest were sample type, sampling period, mucositis, chemotherapy, concurrent antibiotic treatment, neutropenia, inflammation, pain, LLLT and the use of antimicrobial and antimycotic mouth rinses. The third aim was to measure the extent of recovery of the microbiome and therefore the oral microbial community was studied at least 4 weeks after finishing chemotherapy.

2. Materials and methods

2.1 Subjects

Five patients, aged 8-15 years and treated for hematological malignancies, were included in this study after informed consent was obtained from their parents (Ethical approval from Ghent University hospital, Belgian Registration number B670201112526). Patient details and their chemotherapeutic courses during this study period are summarized in Table 5.1. All patients received alkylating agents (cyclophosphamide, etoposide) and anthracyclines (doxorubicin, mitoxantrone, liposomal daunorubicin) known to induce bone marrow aplasia and mucositis. Moreover, some patients (patients 2,3,4) were treated with high dose methotrexate (3 g/m² or 8 g/m²) and cytarabine was administered to patient 5. Prior to chemotherapy, a first sampling was conducted (= Pre-treatment). Next, oral samples were collected once or twice a week over a 2-2.5 month period. In this period patients received two or three chemotherapeutic courses as depicted in Table 5.1. Samples were linked to treatment periods, each starting at the first day of a chemotherapy course and ending when the next chemotherapy course started (= Treatment period 1, 2, 3). Finally, a follow-up sample was collected 1 to 3 months after the last chemotherapeutic treatment. Age and gender-matched children (n=4), without any know systemic disorder were recruited as reference individuals.

2.2 Oral samples

At each sampling point, three sample types were collected: saliva, a buccal swab and a tongue swab. When the patients suffered from mucositis, an extra swab at the lesion was collected. All samples were collected at least 2 hours after eating or brushing teeth and before sampling the oral cavity of the individuals was flushed with drinking water. For the buccal and tongue samples, a sterile cotton swab was gently wiped ten times along the inner cheek or on the dorsal side of the tongue. For the lesion samples, a sterile cotton swab was gently wiped along the lesion. Samples were stored at -20°C prior to DNA extraction.

Table 5.1 - Patient details and chemotherapeutic courses.

COPAD**: Vincristine 2 mg/m²x2; Prednisolone 60 mg/m²/day for 5 days; Cyclophosphamide 250 mg/m²/12 hour x 3 days; Doxorubicin 60 mg/m².** COP**: Cyclophosphamide 300 mg/m²; Vincristine 1 mg/m²; Prednisolone 60 mg/m²/day for 7 days.** COPADM**: COPAD + High Dose Methotrexate (HD MTX) 3g/m²; intrathecal injection of chemotherapy (IT).** CYM**: HD MTX 3g/m² + Cytarabine 100 mg/m²/day for 5 days + IT.** Ritux**: Rituximab 375 mg/m².** COPADM for group C1 **contains HD MTX 8 g/m² instead of 3 g/m²;** Maint 1=2**: Etoposide 150 mg/m²/day for 3 days; Cytarabine 50 mg/m²/12 hours for 5 days;** Maint 3=4**: Prednisolone 30 mg/m²/12 hours for 5 days orally; Cyclophosphamide 500 mg/m²/day for 2 days; Doxorubicin 60 mg/m² on Day1.** MEC**: Mitoxantrone 5 mg/m²/day for 5 days; Etoposide 150 mg/m²/day for 5 days; Cytarabine 200 mg/m²/day for 7 days; IT.** ADxE**: Cytarabine 2x 100 mg/m²/day and 6 x 200 mg/m²/day; liposomal daunorubicin 60 mg/m²/day for 3 days; Etoposide 150 mg/m²/day for 3 days; IT.**

	Age	Gender	Hematological malignancy	Protocol	Treatment period 1	Treatment period 2	Treatment period 3	Treatments after treatment period 3 and before follow-up	# sampling points
Patient 1	8 year	Male	Burkitt lymphoma, stage 2	LMB 2001, group A	COPAD	COPAD	/	/	11
Patient 2	10 year	Male	Burkitt lymphoma, stage 3, high risk	Inter-B-NHL Ritux 2010, group B	COP - COPADM	COPADM	CYM	CYM + Ritux	13
Patient 3	15 year	Male	Diffuse Large B-cell lymphoma (DLBCL)	LMB 2001, group B	COP - COPADM	COPADM	CYM	CYM	14
Patient 4	15 year	Female	Burkitt leukemia	Inter-B-NHL Ritux 2010, group C1	COP COPADM+Ritux	COPADM+Ritux	/	Ritux – Maint1 – Ritux – Maint2 – Maint3 – Maint4	14
Patient 5	11 year	Female	Acute myeloid leukemia	NOPHO DBH AML2012	MEC	ADxE	/	/	14

2.3 Clinical records

Several parameters were registered during the time of the collection of the samples. First, the World Health Organization (WHO) grading scale for oral mucositis was used to estimate the severity of mucositis (WHO 1979). The value of chemotherapy load was determined by increasing the load with one value for each day of subsequent treatment, followed by decreasing the load with one value on each subsequent day without treatment till it reached zero. The same system was used to calculate an antibiotic load, although here the value depended on the category of the antibiotic, based on the antimicrobial spectrum. Patients 2, 3, 4 and 5 were treated with sulfamethoxazole and trimethoprim in the weekend, as prophylaxis against *Pneumocystis jiroveci* infection. On these days, the antimicrobial load increased with a value of 1. The patient with AML (patient 5) got prophylactic treatment with oral ciprofloxacin after each course. Due to its broader antimicrobial spectrum, this was set as category 2. If the patient developed neutropenic fever, broad-spectrum antibiotics were started for empirical treatment of Gram-negative, Gram-positive +/- anaerobic bacteria: either amikacin and ceftriaxone in case of low-risk infection or amikacin and piperacillin/tazobactam in case of profound neutropenia and high risk infection. Amikacin and ceftriaxone were considered as category 2 (value of 2 for each day of administration), and the combination of amikacin and piperacillin/tazobactam had a value of 3. If the fever persisted despite the antibiotics mentioned before or in case of a poor general condition, vancomycin or teicoplanin was added and/or antibiotics were switched to meropenem and ciprofloxacin, which is a combination with a very broad spectrum and therefore all categorized with a value of 4. Patient 5 was treated at the end of the sampling period with amoxicillin with clavulanic acid due to appendicitis. In combination with the prophylactic ciprofloxacin, this was classified in category 3. The use of antibacterial mouth rinses with chlorhexidine (0.05 % or 0.12 %), antimycotic mouth rinses with nystatin (100 000 IE/mL) and treatment with LLLT was monitored. Neutropenia was categorized as mild when neutrophils were between 1000-2500/µL, as moderate when neutrophils were between 500-1000/µL and as severe neutropenia in case of less than 500 neutrophils per µL (National Cancer Institute 2009). The C-reactive protein (CRP) levels were monitored as an inflammatory marker, with mild inflammation between 5-40 mg/L, an active inflammation and bacterial infection (=’moderate’) between 40-200 mg/L and a severe bacterial infection when > 200 mg/L (Clyne and Olshaker 1999). Oral pain was measured using the Visual Analogue Scale (VAS).

2.4 Microbial community analysis

Total DNA was extracted from the buccal or tongue swab or the pellet of 100 µL of saliva sample, as described in Chapter 3, section 2.4. On all samples, DGGE was performed using the 338F-GC and 518R primers targeting the V3 region of the 16S rRNA gene as described in Chapter 3, section 2.5.1. Illumina sequencing of the V3-V4 region of the 16S rRNA gene was performed on saliva, buccal and lesion samples by LGC Genomics (Berlin, Germany) on the MiSeq platform as described in Chapter 3, section 3.5.2.

2.5 Statistical analysis

All statistical analyses were performed in R (version 3.3.2). The packages phyloseq (McMurdie and Holmes 2013) and vegan (Oksanen 2016) were used for microbial community analysis. Heatmaps were generated with the pheatmap package and order-based Hill's numbers (Hill 1973) were calculated. NMDS plots of the bacterial community data were created based on the Bray-Curtis distance measures. Significant differences were identified by means of Permutational ANOVA (PERMANOVA) using the *adonis* function (vegan). Multivariate homogeneity of dispersion (variance) was calculated with the *betadisper* function (vegan), a multivariate analogue of the Levene's test for homogeneity of variances, and significant differences were identified with the Kruskall-Wallis Rank Sum test.

For all other basic statistics, differences between three or more groups were defined via ANOVA and Tukey as post-hoc test, if the data were normally distributed (tested with Shapiro-Wilk test) and homoscedastic (tested with Levene test), if not, Kruskall Wallis Rank Sum test with Tukey post-hoc testing was used as a non-parametric alternative. For the comparison between two groups, a t-test was used for normally distributed and homoscedastic data and the Wilcoxon Rank Sum test as a non-parametric alternative. Differences were considered significant at $p<0.05$.

3. Results

3.1 Oral mucositis

All 5 patients included in this study developed oral mucositis over time, however not all to the same extent. Patient 1 developed only grade 1 mucositis; patient 2 and 3 developed grade 1 and 2; whereas patient 4 and 5 had episodes of grade 1, 2 and 3 mucositis. This is not surprising since the latter patients got the most intensive treatment with 8 g/m² methotrexate and cytarabine on top of anthracyclines, respectively (Table 5.1). Mucositis started between the last days of the course up to 6 days following the chemotherapy, and the duration of mucositis was 2-16 days. Except for patient 1 (the least intensively treated patient),

mucositis always occurred during a period of neutropenia and also often co-occurred with increased C-reactive protein (CRP) levels.

3.2 Oral microbial composition changes over time, but recovers to its initial composition at the follow-up

Oral microbial community analysis of 5 pediatric patients treated for hematological malignancies was assessed by Illumina sequencing of the 16S rRNA gene. Saliva, buccal swabs and lesion samples were analyzed on 11-14 time points over a 2-2.5 month period. Altogether, the seven most abundant phyla (in decreasing order of importance) Firmicutes, Fusobacteria, Proteobacteria, Bacteroidetes, Actinobacteria, Candidatus Saccharibacteria and Tenericutes, comprise at least 97.7 % of abundance in each sample. The most abundant genera were *Streptococcus*, *Veillonella*, *Haemophilus, Fusobacterium*, *Prevotella* and *Neisseria*. As expected, inter-individual variability was large and it was the most important factor explaining the variance in the data (17.1 %, based on Bray-Curtis dissimilarities) (Figure 5.1A). A higher degree of community variation in function of time was observed for patients 4 and 5 ($p=0.0024$) (Figure 5.1B). Similarly, samples during treatment showed an increased community variation compared to both follow-up samples ($p=0.02$) and samples before start of chemotherapy ($p=0.0039$) (Supplementary Figure 5.1). The variation of the community was also significantly larger for samples taken in a period with an antibiotic load ($p=0.0002$), during antibiotic treatment ($p=0.00098$), inflammation ($p=0.0012$), neutropenia ($p=0.0057$) and mucositis ($p=0.0007$) and when using a chlorhexidine mouth rinse ($p=0.0062$), antimycotic mouth rinse ($p=0.0046$) and LLLT ($p=0.017$) (Supplementary Figure 5.1). This indicates that all these factors were related to the increased variation in the microbial composition during treatment.

For each patient, the salivary and buccal composition was followed over time using NMDS based on the Bray-Curtis dissimilarities (Figure 5.2). For patient 1, the salivary microbial composition only changed in treatment period 2, but returned to the initial composition at the follow-up. The buccal samples were more variable over time but the composition of the follow-up samples were also similar to that at the beginning of treatment 1. Similar trends were seen for patient 2, with a salivary microbial community that was stable at the start, changed just before the start of the second chemotherapeutic treatment, but recovered to the initial composition in the end. The buccal and salivary samples from patient 3, were variable during treatment 1 and 2, more constant following treatment 3 and also returned to the initial composition at the follow-up. For patient 4, the buccal and salivary microbial communities were variable during both treatment 1 and 2, but also recovered to the initial microbial composition at the follow-up. However, the buccal microbial community at the follow-up was more similar

to that of the salivary initial composition. For patient 5, the buccal and salivary microbial community were similar at the start and displayed similar changes over time. As no follow-up sample was available for patients 5, no conclusion could be made about recovery.

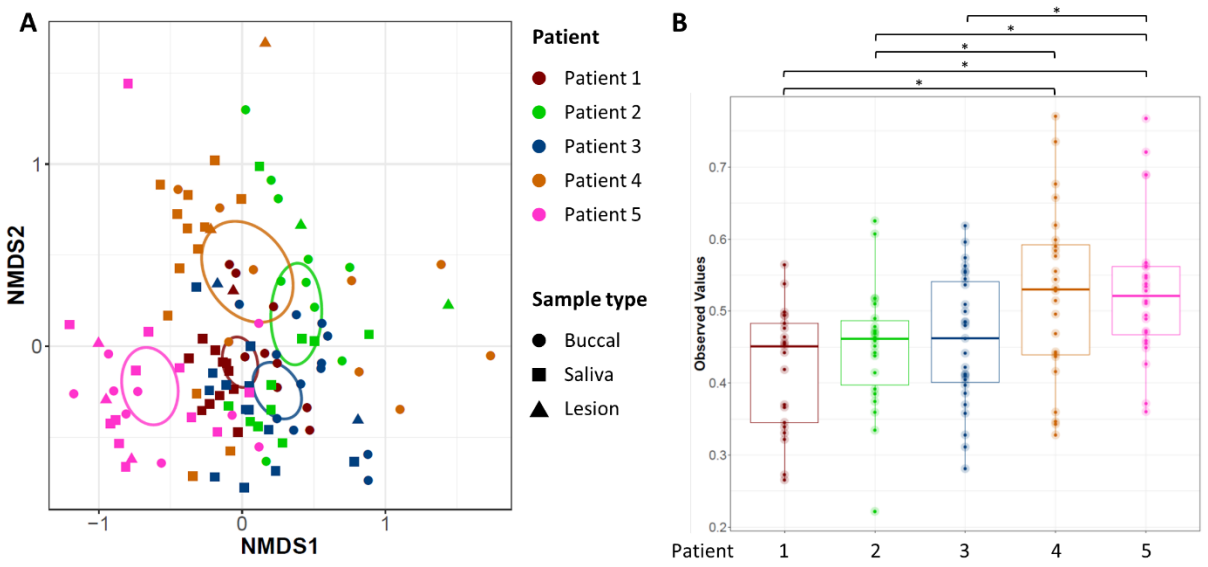


Figure 5.1 - A) NMDS plot of all samples based on Bray-Curtis dissimilarities with 95 % confidence ellipsoids of the mean; B) Distance to the centroid, based on Bray-Curtis dissimilarities. Significant differences between groups are indicated by the asterisks (p<0.05).

3.3 Diversity decreased following chemotherapeutic and antibiotic treatment and partially recovered at the follow-up

The α-diversity of the samples was assessed with the inverse Simpson index, also known as the second Hill number (Hill 1973). Overall, no significant differences were observed between diversity of saliva versus buccal samples (p=0.26). For both saliva and buccal samples, diversity changed over time showing similar profiles. Minor decreases could be observed after a chemotherapy course, whereas diversity largely decreased during or following antibiotic treatment (Figure 5.3). Similar trends were observed for the Hill numbers representing richness and evenness (data not shown). At the follow-up (at least 1 month following the last chemotherapeutic treatment) only a partial recovery of both buccal and salivary bacterial diversity was observed (Figure 5.3).

Diversity was also measured based on the DGGE microbial profiles of saliva, buccal, tongue and lesion samples (Supplementary Figure 5.2; DGGE profiles see Supplementary Figure 5.3 and Supplementary Figure 5.4). Diversity profiles showed similar trends as compared to Illumina sequencing results i.e. decreases in diversity following chemotherapeutic and antibiotic treatments. The DGGE profiles of four healthy children (Supplementary Figure 5.5) showed that salivary diversity of pre-treatment and follow-up samples of the patients (19.5 ± 7.1) was similar to that of healthy children (18.2 ±7.5).

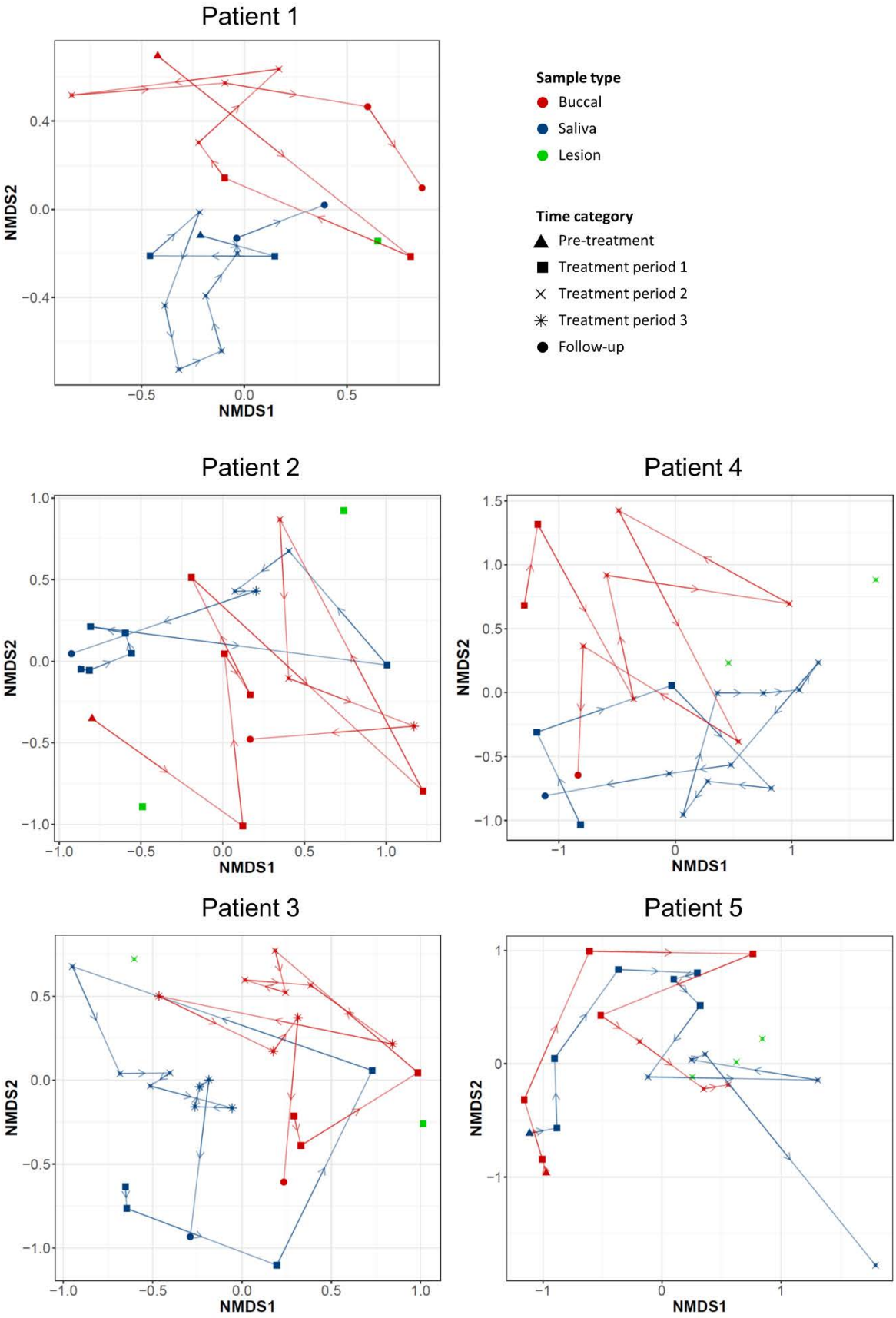


Figure 5.2 - NMDS plots based on Bray-Curtis dissimilarities indicating the shifts in microbial community over time. Consecutive time point are connected.

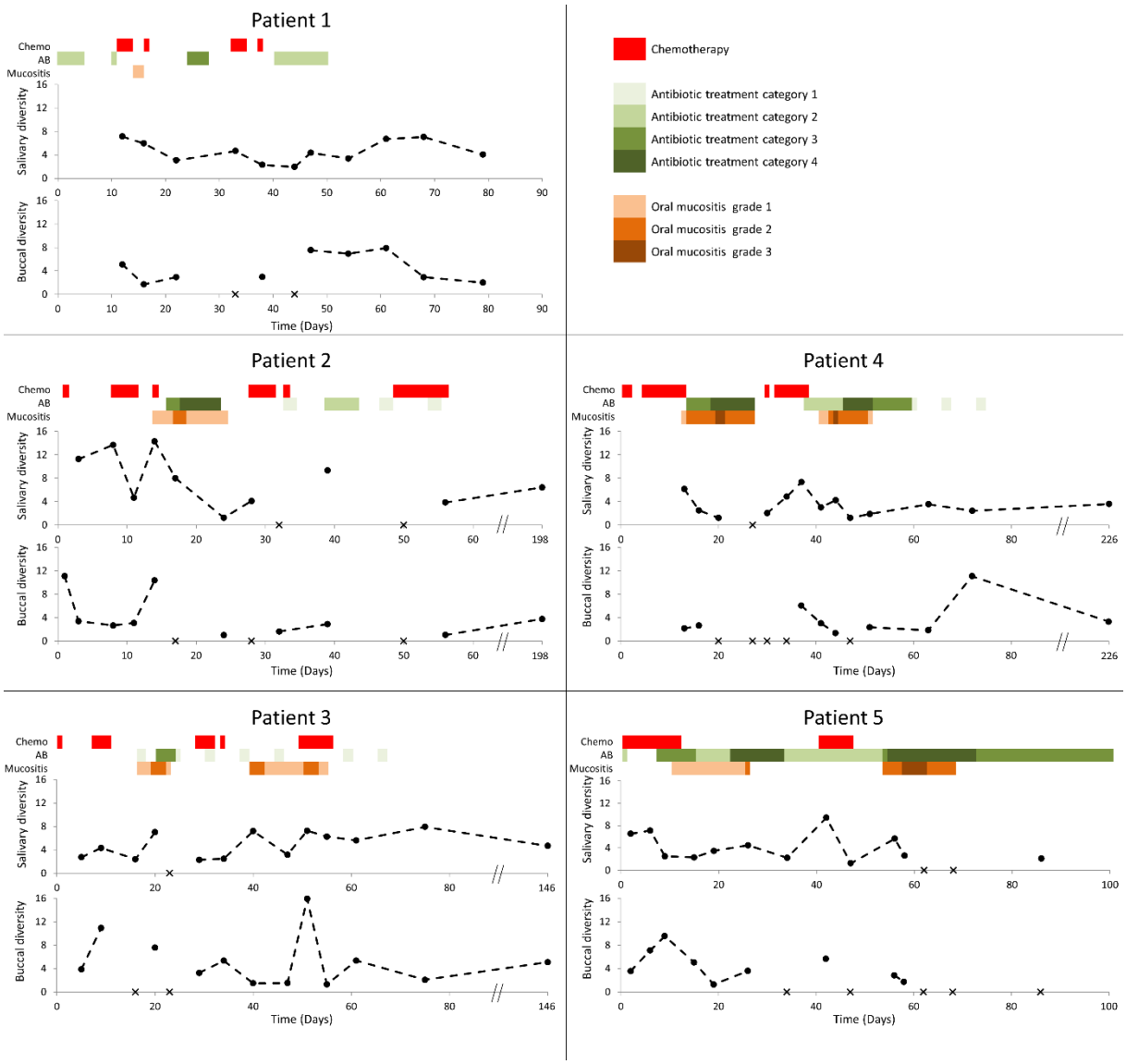


Figure 5.3 - Buccal and salivary diversity (Hill number 2 = inverse Simpson coefficient) based on Illumina amplicon sequencing data. Data with less than 1000 reads per sample are indicated with a 'X'.

3.4 Multiple confounding factors determine the oral microbial composition

Metadata such as chemotherapeutic treatment, mucositis, pain, antibiotic treatment, use of antimicrobial or antimycotic mouth rinses, LLLT, neutropenia, and inflammation were monitored during the sampling period (Supplementary Table 5.1). All factors correlated significantly with the overall microbial community variation based on Bray-Curtis dissimilarities, with the individual (17.1 %), sample type (9.0 %), antibiotic grade (7.7 %), inflammation grade (6.9 %) and sampling period (6.3 %) as five most important factors (Table 5.2). As large inter-individual variability was observed, the most important confounding factors were also determined for each patient separately. Some variability is reported among the five most important confounding factors for each patient, but some general trends can be noticed (Table

5.2). Similar to the analysis of the entire dataset, the sample type (saliva vs. buccal vs. lesion) and the period of sampling (pre-treatment vs. treatment period 1 vs. 2 vs. 3 vs. follow-up) explained a high percentage of the variation for each of the five patients (respectively 10.8-28.5 % and 17.2-31.1 %). Next to the antibiotic grade, also the antibiotic load was an important confounding factor. They each had a significant contribution in 2 and 4 of the patients respectively. In those cases, they explained respectively 12.2-15.8 % and 9.2-13.5 % of the variation. The grade of inflammation was highly important for patient 4 and 5 (17.7 % and 26.1 %), whereas also the grade of neutropenia was an important cofactor for 4 out of 5 patients (13.4-22.7 %). Although the use of an antibacterial mouth rinse with chlorhexidine only explained 3.7 % of the variation of the entire dataset, it significantly explained 11-20.1 % of the variation in the microbial community for all 4 patients who used it. This may indicate patient-specific changes due to oral mouth rinse. Mucositis, chemotherapy and chemotherapy load were ranked in the top-five confounding factors for only 1 patient each. Pain and the use of LLLT and antimycotic rinse were less important confounding factors as they were not ranked in the top-five for any patient.

Table 5.2 - R^2 (%) based on the Bray-Curtis dissimilarities for the entire dataset (ALL) and for each patient (P1-P5). Significant differences are indicated in red. The five largest values per column are indicated with a grey background.

(%)	ALL	P1	P2	P3	P4	P5
Patient	17.1	*NA*	*NA*	*NA*	*NA*	*NA*
Sample type	9.0	28.5	23.9	22.7	10.8	14.4
Sampling period	6.3	31.1	20.7	20.3	17.2	27.7
Chemotherapy	1.7	3.8	2.8	6.8	14.9	13.3
Chemotherapy load	1.9	3.8	2.8	6.0	5.7	20.9
Antibiotic grade	7.7	9.0	3.3	*NA*	12.2	15.8
Antibiotic load	4.7	12.8	11.7	7.7	9.2	13.5
Antibacterial mouth rinse	3.7	17.2	15.0	11.0	*NA*	20.1
Antimycotic mouth rinse	2.7	9.2	4.9	8.0	11.8	8.0
Low level laser therapy	2.6	*NA*	4.5	8.0	12.5	12.2
Mucositis grade	4.5	11.3	4.2	7.1	9.0	17.0
Neutropenia grade	5.2	15.5	22.7	13.4	17.0	7.2
Inflammation grade	6.9	8.5	8.3	5.3	17.7	26.1
Pain	2.5	11.3	10.3	4.3	4.8	16.1

3.5 Patient-specific shifts at genus level and increases in opportunistic pathogens

Heatmaps of the most abundant genera (average abundance > 1 % or present in one sample with an abundance > 5 %) indicated that the shifts occurring over time were patient-specific (Figure 5.4). For patient 1, both buccal (50-76 %) and saliva (35-41 %) samples started with high abundances of *Streptococcus*, which decreased (to minimum 5 %) following the first and second chemotherapeutic course. Relative abundances increased again in the follow-up samples to initial levels. An inverse trend was observed for *Veillonella* in saliva samples, with an initial increase in abundance (till maximum 70 %), followed by a decrease in the follow-up samples. The buccal sample at day 38 was dominated by *Gemella* (51 %). The lesion sample had a large *Streptococcus* abundance (62 %), similar to the buccal sample of that day.

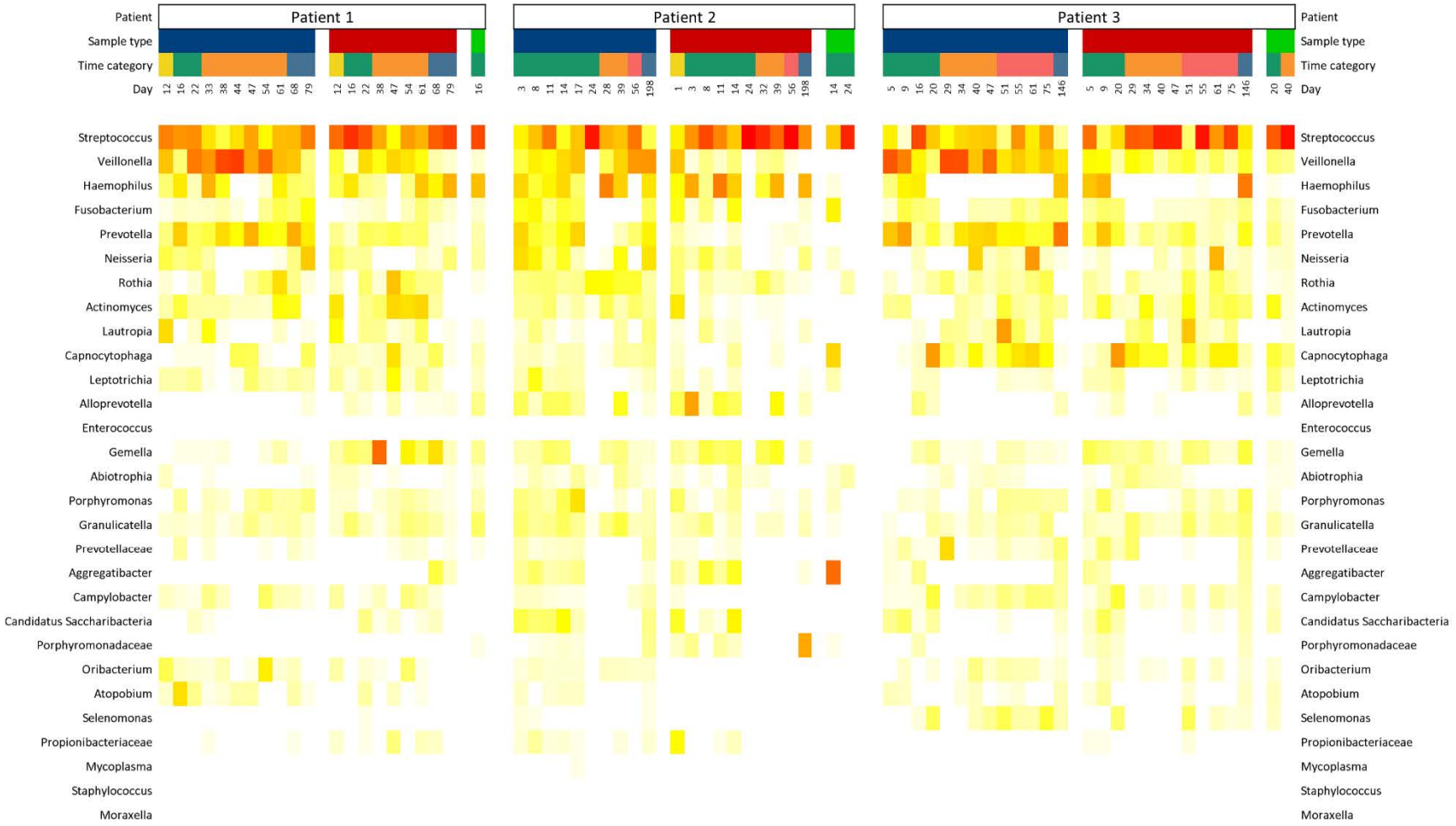
The microbial community of patient 2 showed no clear shifts during the first three weeks. At day 24 however, a significant change occurred in both the saliva and buccal community, with *Streptococcus* almost completely dominating the community (90-99 %). In the buccal community, *Streptococcus* dominated for even longer, although in the saliva the abundance largely decreased at the next sampling point. Lesions were dominated by *Aggregatibacter* (50 %, day 14) or *Streptococcus* (81 %, day 24).

The oral microbial profile of patient 3 was similar to patient 2, with *Streptococcus* dominating the buccal samples (34-91 %) from the start of the second treatment period. In saliva samples, high abundances of *Veillonella* were observed both in the pre-chemotherapy sample as during and following the second chemotherapeutic course. Both in saliva and buccal samples, *Haemophilus* was present in the initial samples, but completely disappeared after the first chemotherapeutic treatment, yet re-occurred at the follow-up at high abundances (22-44 %). *Capnocytophaga* was present at day 20 at higher abundances (33-35 %), whereas *Lautropia* was highly present at day 51 (21-33 %). Lesions were dominated by *Streptococcus* (57-93 %).

For patient 4, each sample was dominated by a different genus. Starting with *Haemophilus* at day 13 (35-68 %), *Lautropia* at day 16 (49-57 %), *Abiotrophia* at day 20 (90 %), *Rothia* at day 30 (67 %), *Enterococcus* at day 44 (buccal 85 %) and day 47 (lesion 99 %), *Streptococcus* at day 47, 51 and 53 (54-91 %), and *Veillonella* at day 72 (saliva 62 %). Lesions were dominated by *Enterococcus* (day 47, 99 %) or *Enterococcus* and *Streptococcus* (day 44, 26 % and 37 % respectively).

A similar shifting dominance was observed for the samples from patient 5. The first sampling points were characterized by a high diversity, but *Veillonella* dominated at day 9 and 15 (saliva 62-65 %), *Leptotrichia* at day 19 (59-89 %), *Porphyromonas* at day 26 in saliva (41 %), *Actinomyces* at day 26 in buccal swab (52 %) *Fusobacterium* at day 34 and 58 in saliva

(63 and 52 %) and day 56 and 58 in buccal samples (57 and 75 %) and *Neisseria* at day 47 and 86 in saliva (89 and 50 %). In contrast to the other donors, *Streptococcus* almost disappeared from day 19 till day 62. Samples derived from lesions were dominated by *Fusobacterium* (49, 70 and 84 %).


Patient
Sample type
Time category
Day
Patient 1
Patient 2
Patient 3
12 16 22 33 38 44 47 54 61 68 79 12 16 22 38 47 54 61 68 79 16
3 8 11 14 17 24 28 39 56 198 1 3 8 11 14 24 32 39 56 198 14 24
5 9 16 20 29 34 40 47 51 55 61 75 146 5 9 20 29 34 40 47 51 55 61 75 146 20 40
Streptococcus
Veillonella
Haemophilus
Fusobacterium
Prevotella
Neisseria
Rothia
Actinomyces
Lautropia
Capnocytophaga
Leptotrichia
Alloprevotella
Enterococcus
Gemella
Abiotrophia
Porphyromonas
Granulicatella
Prevotellaceae
Aggregatibacter
Campylobacter
Candidatus Saccharibacteria
Porphyromonadaceae
Oribacterium
Atopobium
Selenomonas
Propionibacteriaceae
Mycoplasma
Staphylococcus
Moraxella

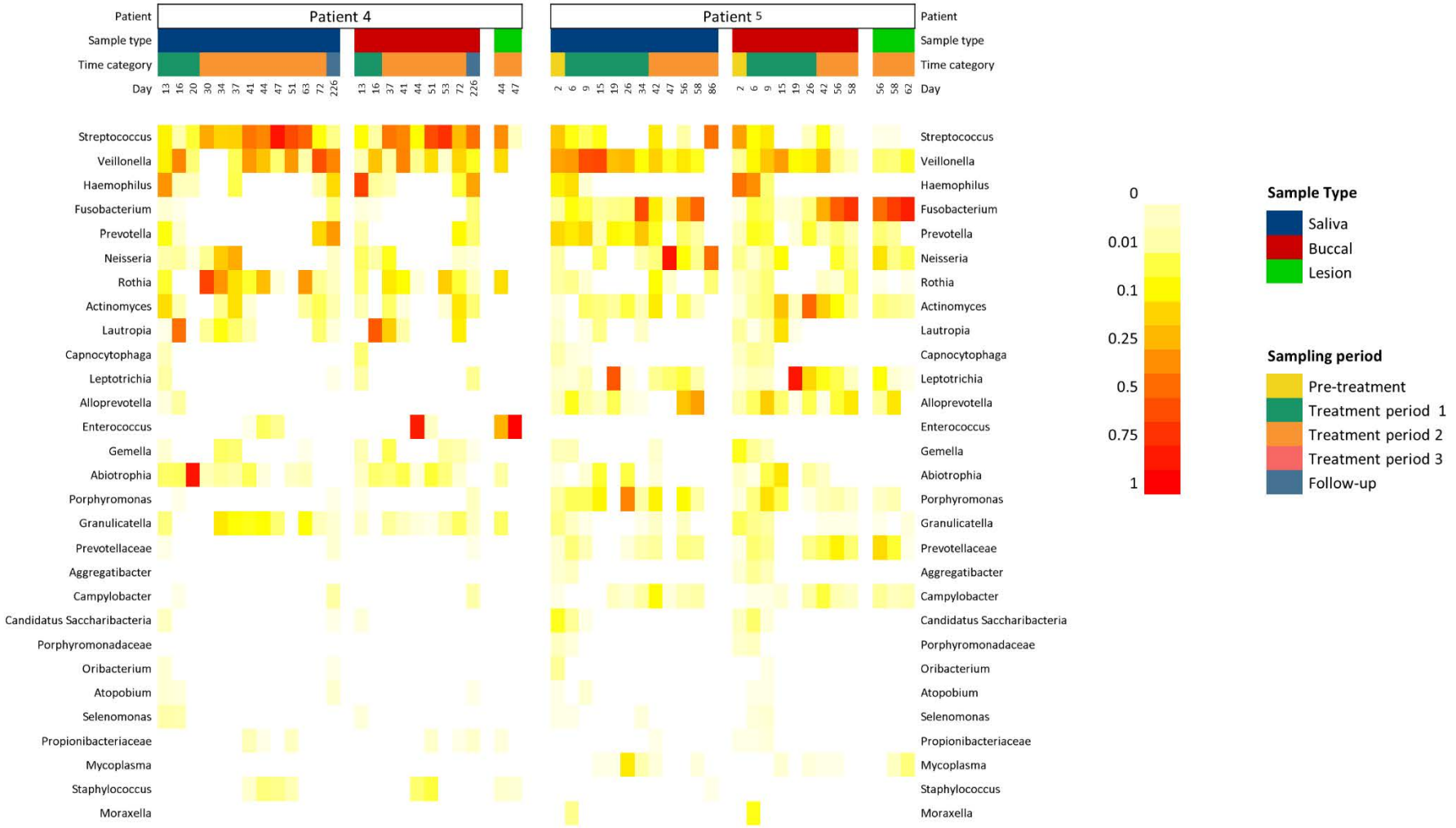


Figure 5.4 - Heatmaps representing the genera with average abundance > 1 % or present in at least 1 sample > 5 %.

4. Discussion

Longitudinal monitoring of five pediatric cancer patients revealed a large degree of inter-individual variability in microbial community composition and dynamics. Although Wang et al. (2014) showed that disease-related shifts in the oral microbiome occur prior to chemotherapy in AML patients, we did not find a deviating oral microbiome structure of the five patients prior to chemotherapy as opposed to that of healthy children. However, profound microbiome shifts occurred for all patients during and following the chemotherapy courses over this long-term period. This was particularly noticed for two study patients (patient 4 and 5) who displayed the highest degree in community variation over time. These two patients were also subjected to the most toxic chemotherapy courses and highest antibiotic loads and they experienced the most severe mucositis grade. Independent of sample type (saliva vs. buccal) major shifts in dominant oral genera, such as *Fusobacterium*, *Neisseria*, *Rothia*, *Actinomyces*, *Lautropia*, *Leptotrichia*, *Enterococcus* and *Abiotrophia* were noted for these two patients, primarily after antibiotic treatment. Yet, we were unable to reveal consistent shifts in microbiome composition across the different individuals, indicating interindividual microbiome differences to be a predominant factor in explaining dataset variability.

Although profound shifts were observed over time, the microbial community recovered to its initial composition in the follow-up samples, 1 to 3 months after the last chemotherapeutic treatment. Despite this recovery in microbial community composition, ecosystem diversity only partially recovered at the follow-up. As microbial diversity closely correlates with functional diversity (Huttenhower et al. 2012), a lower diversity may indicate an ecosystem to be less homeostatic and closer to an ecological tipping point (Scheffer et al. 2009, Wittebolle et al. 2009). Low diversity in combination with disrupted community composition may predispose for outgrowth of one species dominating the entire oral microbiota. This may eventually result in increased infection risks, as has also been reported for AML patients (Galloway-Pena et al. 2017). These data underline the importance of maintaining a homeostatic microbiome and limiting microbiome shifts during treatment. This could be obtained through long-term proper oral hygiene practices and regular oral health care, also after finishing chemotherapeutic treatment.

With respect to the nature of the treatment it was expected that chemotherapy would create clear shifts in the oral microbiome composition. However, chemotherapy only caused limited shifts in community composition in the five patients. In contrast, community diversity did decrease upon chemotherapy, paralleling previous findings of a decreased microbial diversity in fecal samples of AML patients (van Vliet et al. 2009). In addition, the toxic nature of the chemotherapy clearly correlated with the grade of mucositis: yet, despite its profound

impact on the quality of life for the patient, the grade of mucositis was not the most important confounding factor to explain oral microbial shifts for the five patients.

In sharp contrast to chemotherapy and mucositis, antibiotic use was found a major confounding factor as it significantly affected both microbiome composition and diversity. This was also reflected in the high number of samples with low read counts (<1000) that originated from antimicrobial treatment periods. Although most scientific research primarily targets the effect of antibiotics on the gut microbiome, several antibiotics also have been shown to be present in saliva (Troeltzsch et al. 2014) and to impact the oral microbiome (de Vries-Hospers et al. 1991, Brismar et al. 1993, Sullivan et al. 2001, Zaura et al. 2015, Abeles et al. 2016). These include for example amoxicillin, ceftriaxone and ciprofloxacin, which were also used in our longitudinal study. In correspondence with our findings, Galloway-Pena et al. (2017) demonstrated that the duration of antibiotic treatment was significantly associated with an increased temporal variability of oral microbiome diversity and composition of hospitalized cancer patients. Following the above-mentioned lower microbiome diversity upon chemotherapy, we demonstrate that antibiotic use further impacts the already disturbed oral microbial community. This may be considered a risk factor for developing infections and for more severe mucositis episodes. Interestingly, the prophylactic use of antibiotics for the treatment of pediatric AML patients is currently under debate (Gamis 2015). As clinical evidence is lacking or even inadequate (McGuire et al. 2013), MASCC/ISOO guidelines do not support the prophylactic use of chlorhexidine mouth rinse to prevent oral mucositis (Lalla et al. 2014). Our findings seem to support this view: not only systemic antibiotics, but also chlorhexidine-based mouth rinses were major contributors to microbiome disturbances at an individual level. Yet, clinicians could still opt for the use of antimicrobial mouth rinse if a clear association or even causation of specific microorganisms with mucositis would be demonstrated.

We therefore also characterized the microbiome of lesions to reveal specific genera that could possibly associate with mucositis. Confirming previous reports (Ye et al., 2013), *Streptococcus* was the most prominent genus in lesion samples. While this finding may just be indicative of its status as common oral microbe, we also found *Aggregatibacter*, *Enterococcus* and *Fusobacterium* to be highly enriched in lesion samples from some patients. Several of these genera have been associated with pathogenicity (Teles et al. 2013). Yet we could not detect a common mucositis phylotype across the 5 patients. Our data are similar to previous sequencing data of mucositis lesions where high intersample variability was described with *Actinomyces*, *Rothia*, *Prevotella*, *Staphylococcus*, *Abiotrophia* and *Lactobacillus* spp. being abundant next to *Streptococcus* (Ye et al. 2013). Likewise, culture-based techniques did not reveal one common pathogen but rather an association of *Enterococcus*, *Porphyromonas*, *Staphylococcus* or *Fusobacterium* with mucositis and oral ulcers (Laheij et al. 2012, Olczak-

Kowalczyk et al. 2012). Based on our observations we concur with Ye et al. (2013) that mucositis risk and severity is determined by the entire community, rather than an association with one specific pathogen.

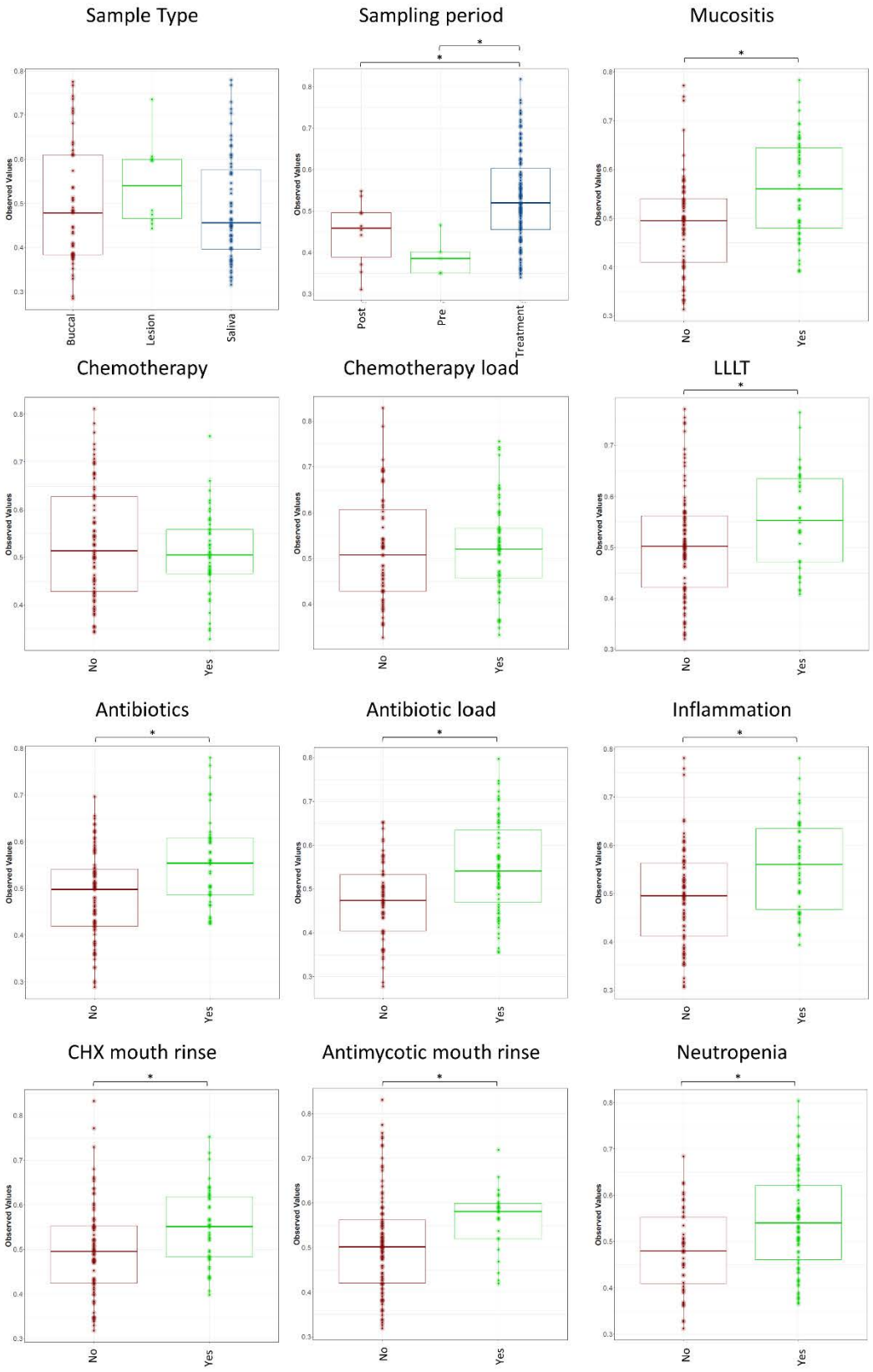
Similar to previous studies, buccal samples were characterized by a higher *Streptococcus* abundance (Segata et al. 2012). Dominance of *Streptococcus* was observed in samples from patient 1, 2 and 3. In contrast, *Streptococcus* almost disappeared from the oral cavity of patients 5 from day 15 till day 58. This co-occurred with the use of vancomycin mouth spray, targeting Gram-positive species. Despite this very low oral *Streptococcus* abundance, a positive *Streptococcus viridans* hemoculture (day 54) was observed during this period. For none of the other patients a *Streptococcus* sepsis was reported, although they had high abundances of streptococci in the oral cavity during mucositis periods. This indicates that the use of vancomycin as an oral mouth rinse eradicates streptococci, but does not prevent *Streptococcus* sepsis, which contradicts previous research (Brunet et al. 2006).

In conclusion, longitudinal follow-up of pediatric patients with hematological malignancies revealed chemotherapy and mucositis to have a minor contribution to oral microbiome shifts. Yet, we hypothesize that the observed decrease in microbiome diversity may bring the oral microbiome closer to an ecological tipping point, resulting in a higher susceptibility to severe community disruption, once antibiotic treatment and/or antibacterial mouth rinses are given. The resulting dysbiosis could increase the risk of infection and bacterial sepsis, further aggravating mucositis episodes to which these patients are highly vulnerable. Our findings therefore support current MASCC/ISOO guidelines which do not support the prophylactic use of antimicrobial mouth rinses to prevent chemotherapy-induced oral mucositis.

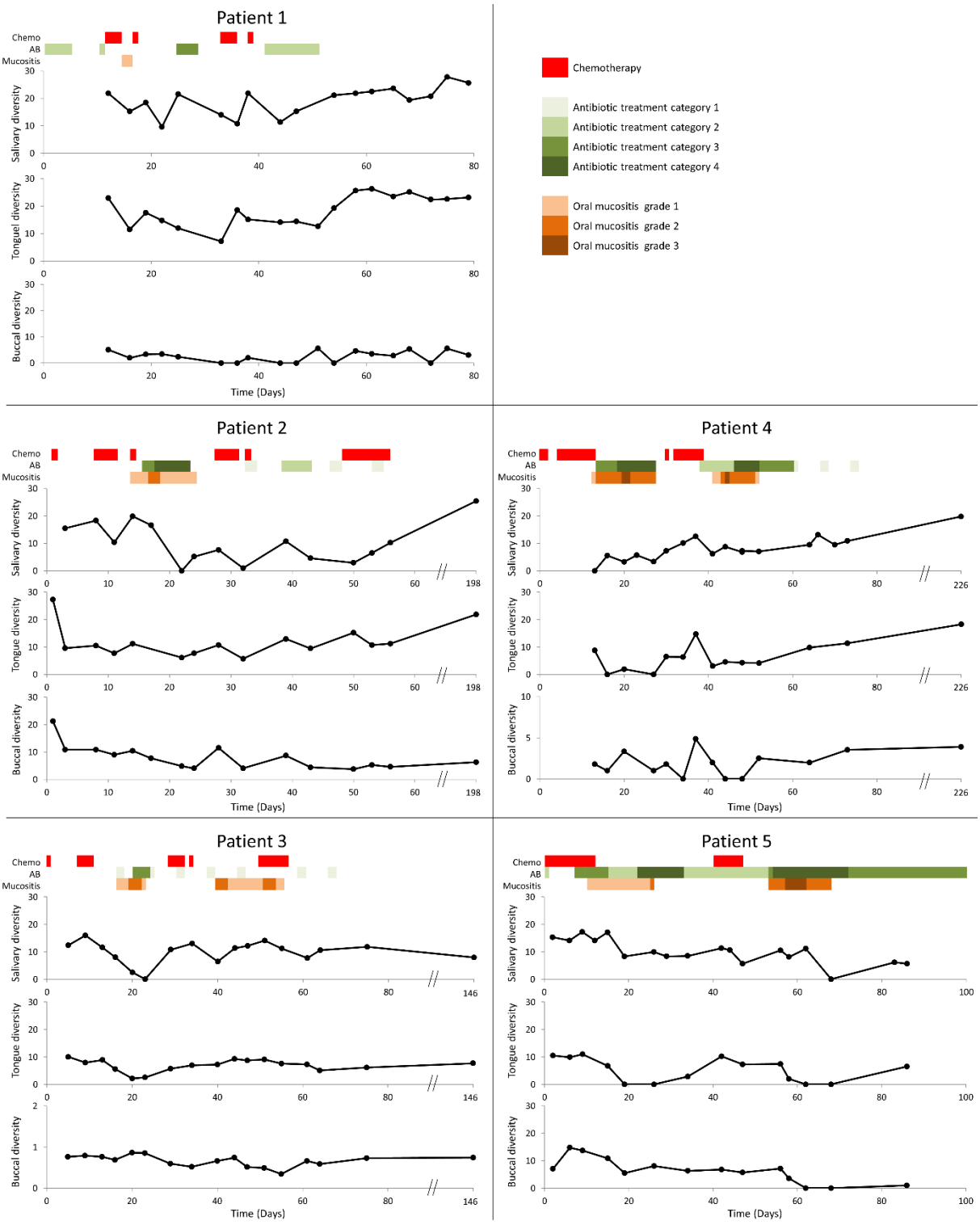
5. Acknowledgements

E.V. is a doctoral research fellow supported by the Bijzonder Onderzoeksfonds (Ghent University; BOF13/DOC/280). This research was funded by BOF17/GOA/032 from Ghent University. J.D.V. is supported as postdoctoral fellow from the Research Foundation Flanders (FWO-Vlaanderen). B.V. was funded by the Seventh Framework Programme (FP7/2011) under grant agreement n° 299169 (Mucositis Platform). The authors would like to thank Maaike Van Hoecke, Rein Demuynck, Annelore Dehoorne and Véronique Van de Velde for the collection of the samples.

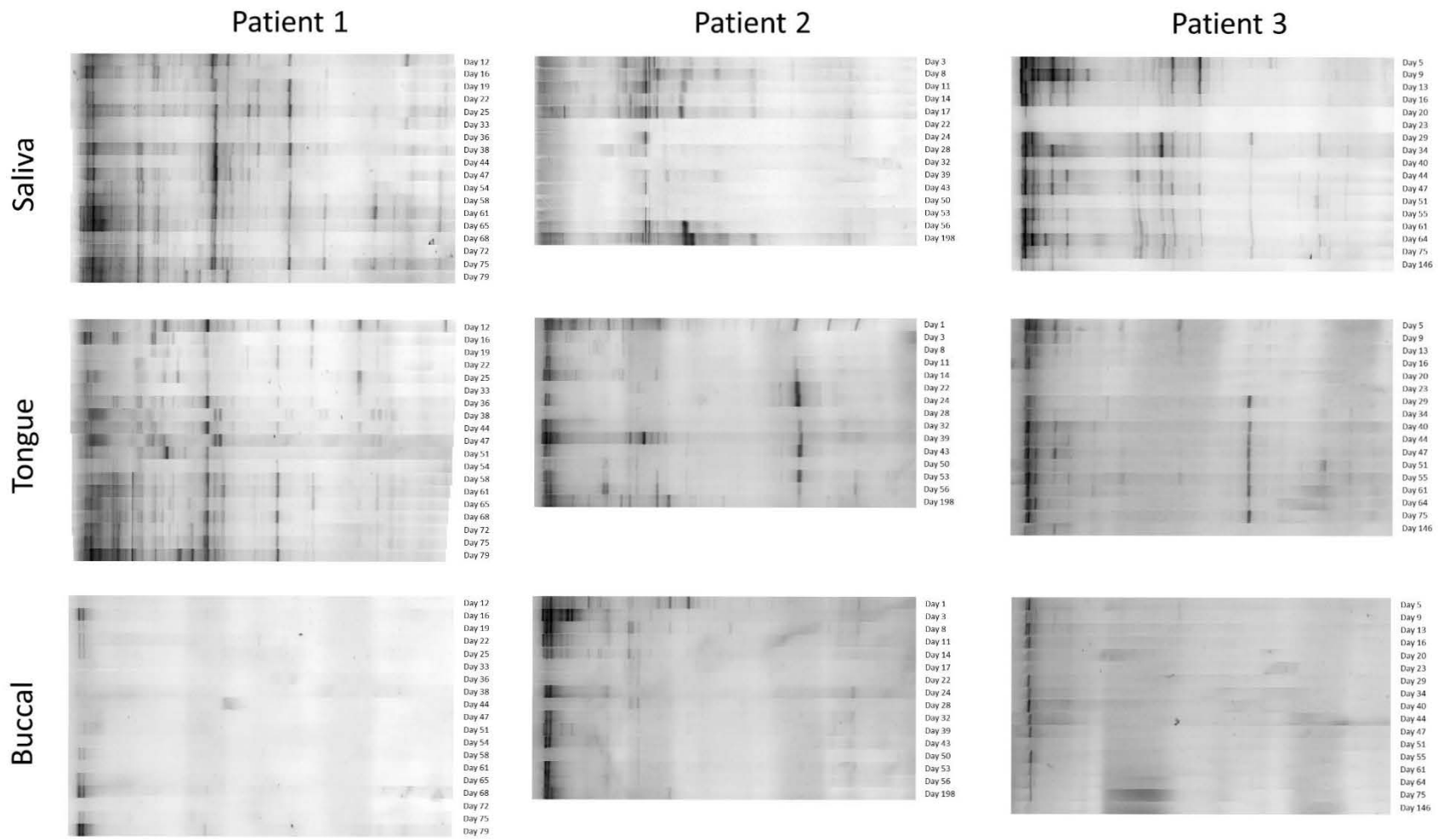
6. Supplementary information



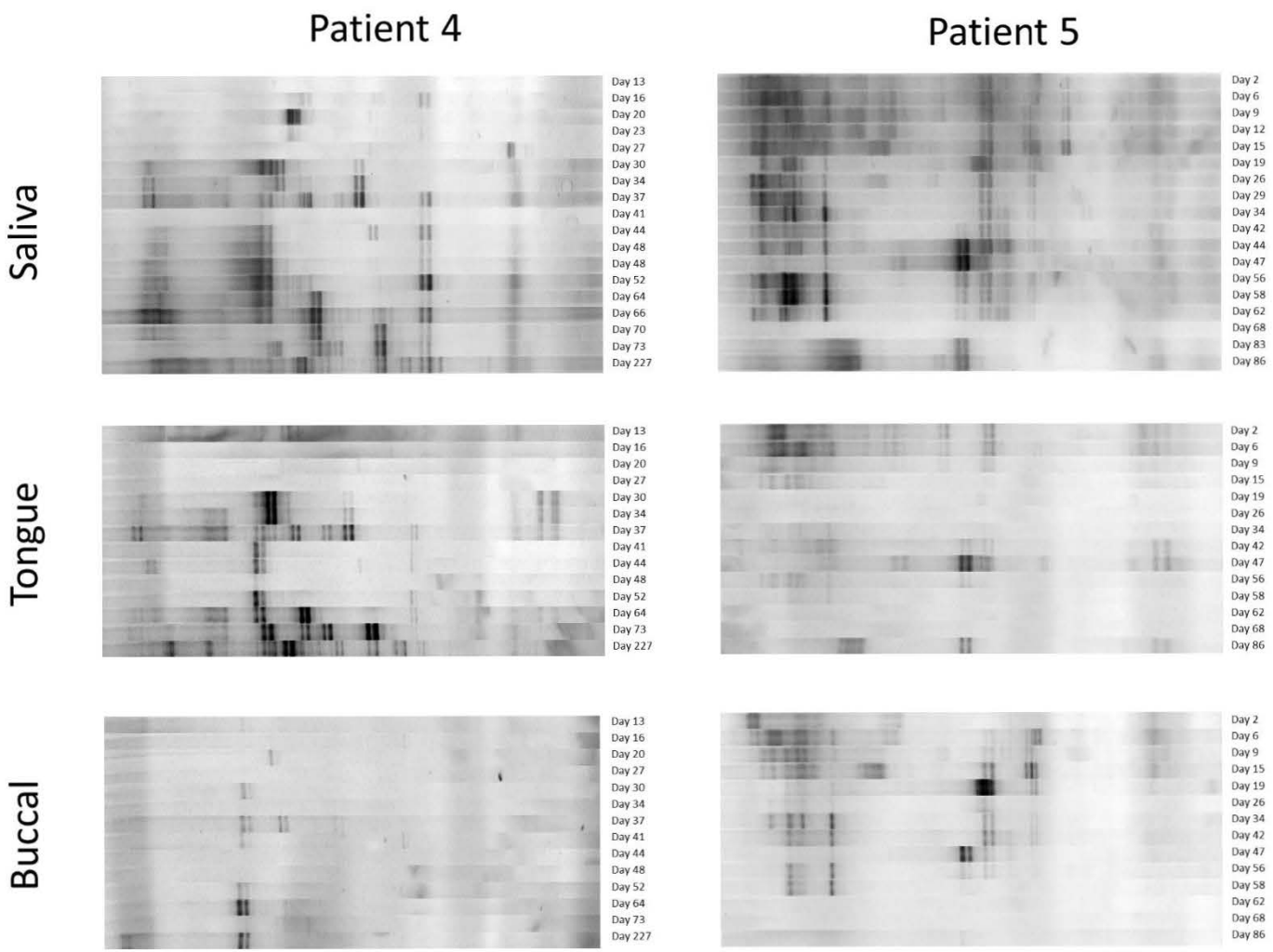
Supplementary Figure 5.1 - Distance to the centroid, based on Bray-Curtis dissimilarities. Significant differences between groups are indicated by the asterisks (p<0.05).



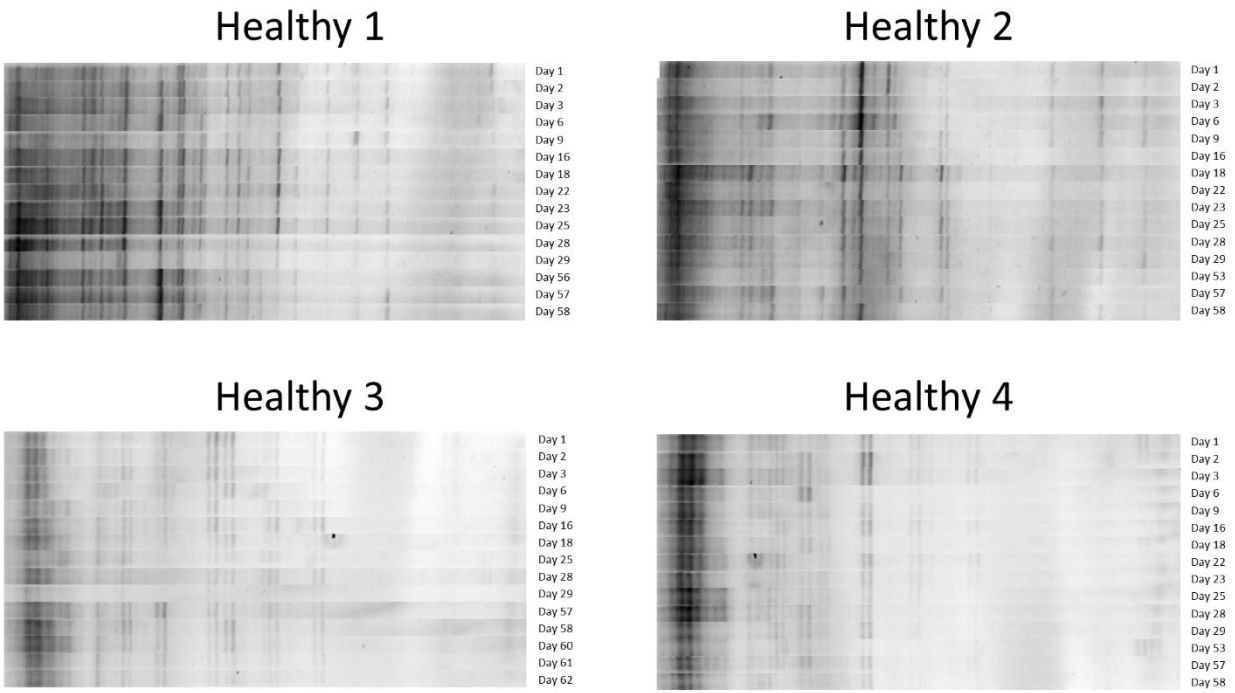
Supplementary Figure 5.2 - Salivary, tongue and buccal diversity (inverse Simpson), based on DGGE.



Supplementary Figure 5.3 - DGGE profiles of saliva, tongue and buccal samples of patient 1, 2 and 3.



Supplementary Figure 5.4 – DGGE profiles of saliva, tongue and buccal samples of patient 4 and 5.



Supplementary Figure 5.5 - DGGE profiles of saliva samples of 4 healthy children.

Supplementary Table 5.1 – Metadata of all oral samples

Patient	Time	Sample type	Sampling period	Chemo	Chemo load	Chemo load Y/N	Antibiotic category	AB Y/N	AB load	AB load Y/N	AB rinse	Antimycotic rinse	LLLT	Mucositis grade	Mucositis Y/N	Neutropenia grade	Neutropenia Y/N	Inflammation grade	Inflammation Y/N	Pain score	Pain Y/N
Patient 1	Day 12	Saliva	Pre-treatment	Yes	1	Yes	AB Category 2	Yes	0	No	No	No	No	Grade 0	No	No	No	No	No	0	No
Patient 1	Day 16	Saliva	Treatment 1	Yes	1	Yes	AB Category 0	No	0	No	No	Yes	No	Grade 1	Yes	No	No	No	No	4	Yes
Patient 1	Day 22	Saliva	Treatment 1	No	0	No	AB Category 0	No	0	No	No	Yes	No	Grade 0	No	Severe	Yes	No	No	0	No
Patient 1	Day 33	Saliva	Treatment 2	Yes	1	Yes	AB Category 0	No	0	No	No	No	No	Grade 0	No	No	No	No	No	0	No
Patient 1	Day 38	Saliva	Treatment 2	Yes	2	Yes	AB Category 0	No	0	No	Yes	No	No	Grade 0	No	No	No	No	No	0	No
Patient 1	Day 44	Saliva	Treatment 2	No	0	No	AB Category 3	Yes	8	Yes	Yes	No	No	Grade 0	No	Moderate	Yes	Mild	Yes	0	No
Patient 1	Day 47	Saliva	Treatment 2	No	0	No	AB Category 3	Yes	14	Yes	Yes	No	No	Grade 0	No	Moderate	Yes	No	No	0	No
Patient 1	Day 54	Saliva	Treatment 2	No	0	No	AB Category 0	No	12	Yes	Yes	No	No	Grade 0	No	Moderate	Yes	Mild	Yes	0	No
Patient 1	Day 61	Saliva	Treatment 2	No	0	No	AB Category 0	No	0	No	No	No	No	Grade 0	No	No	No	No	No	0	No
Patient 1	Day 68	Saliva	Follow-up	No	0	No	AB Category 0	No	0	No	No	No	No	Grade 0	No	No	No	No	No	0	No
Patient 1	Day 79	Saliva	Follow-up	No	0	No	AB Category 0	No	0	No	No	No	No	Grade 0	No	No	No	No	No	0	No
Patient 1	Day 12	Buccal	Pre-treatment	Yes	1	Yes	AB Category 2	Yes	0	No	No	No	No	Grade 0	No	No	No	No	No	0	No
Patient 1	Day 16	Buccal	Treatment 1	Yes	1	Yes	AB Category 0	No	0	No	No	Yes	No	Grade 1	Yes	No	No	No	No	4	Yes
Patient 1	Day 22	Buccal	Treatment 1	No	0	No	AB Category 0	No	0	No	No	Yes	No	Grade 0	No	Severe	Yes	No	No	0	No
Patient 1	Day 33	Buccal	Treatment 2	Yes	1	Yes	AB Category 0	No	0	No	No	No	No	Grade 0	No	No	No	No	No	0	No
Patient 1	Day 38	Buccal	Treatment 2	Yes	2	Yes	AB Category 0	No	0	No	Yes	No	No	Grade 0	No	No	No	No	No	0	No
Patient 1	Day 44	Buccal	Treatment 2	No	0	No	AB Category 3	Yes	8	Yes	Yes	No	No	Grade 0	No	Moderate	Yes	Mild	Yes	0	No
Patient 1	Day 47	Buccal	Treatment 2	No	0	No	AB Category 3	Yes	14	Yes	Yes	No	No	Grade 0	No	Moderate	Yes	No	No	0	No
Patient 1	Day 54	Buccal	Treatment 2	No	0	No	AB Category 0	No	12	Yes	Yes	No	No	Grade 0	No	Moderate	Yes	Mild	Yes	0	No
Patient 1	Day 61	Buccal	Treatment 2	No	0	No	AB Category 0	No	0	No	No	No	No	Grade 0	No	No	No	No	No	0	No
Patient 1	Day 68	Buccal	Follow-up	No	0	No	AB Category 0	No	0	No	No	No	No	Grade 0	No	No	No	No	No	0	No
Patient 1	Day 79	Buccal	Follow-up	No	0	No	AB Category 0	No	0	No	No	No	No	Grade 0	No	No	No	No	No	0	No
Patient 1	Day 16	Lesion	Treatment 1	Yes	1	Yes	AB Category 0	No	0	No	No	Yes	No	Grade 1	Yes	No	No	No	No	4	Yes
Patient 2	Day 3	Saliva	Treatment 1	No	0	No	AB Category 0	No	0	No	No	No	No	Grade 0	No	No	No	Mild	Yes	5	Yes
Patient 2	Day 8	Saliva	Treatment 1	Yes	1	Yes	AB Category 0	No	0	No	No	No	No	Grade 0	No	No	No	No	No	0	No

Patient	Time	Sample type	Sampling period	Chemo	Chemo load	Chemo load Y/N	Antibiotic category	AB Y/N	AB load	AB load Y/N	AB rinse	Antimycotic rinse	LLLT	Mucositis grade	Mucositis Y/N	Neutropenia grade	Neutropenia Y/N	Inflammation grade	Inflammation Y/N	Pain score	Pain Y/N
Patient 2	Day 11	Saliva	Treatment 1	Yes	4	Yes	AB Category 0	No	0	No	No	No	No	Grade 0	No	Severe	Yes	No	No	0	No
Patient 2	Day 14	Saliva	Treatment 1	Yes	3	Yes	AB Category 0	No	0	No	No	No	Yes	Grade 1	Yes	Severe	Yes	No	No	5	Yes
Patient 2	Day 17	Saliva	Treatment 1	No	0	No	AB Category 3	Yes	6	Yes	No	Yes	Yes	Grade 2	Yes	Severe	Yes	Severe	Yes	0	No
Patient 2	Day 24	Saliva	Treatment 1	No	0	No	AB Category 0	No	26	Yes	Yes	No	Yes	Grade 1	Yes	Moderate	Yes	No	No	0	No
Patient 2	Day 28	Saliva	Treatment 2	Yes	1	Yes	AB Category 0	No	10	Yes	No	No	No	Grade 0	No	Mild	Yes	No	No	0	No
Patient 2	Day 32	Saliva	Treatment 2	Yes	3	Yes	AB Category 0	No	0	No	No	No	No	Grade 0	No	No	No	No	No	0	No
Patient 2	Day 39	Saliva	Treatment 2	No	0	No	AB Category 2	Yes	2	Yes	Yes	No	No	Grade 0	No	Severe	Yes	Mild	Yes	0	No
Patient 2	Day 50	Saliva	Treatment 3	Yes	2	Yes	AB Category 0	No	0	No	Yes	No	No	Grade 0	No	Mild	Yes	No	No	0	No
Patient 2	Day 56	Saliva	Treatment 3	Yes	8	Yes	AB Category 0	No	1	Yes	Yes	No	No	Grade 0	No	Mild	Yes	Mild	Yes	0	No
Patient 2	Day 198	Saliva	Follow-up	No	0	No	AB Category 0	No	0	No	No	No	No	Grade 0	No	No	No	No	No	0	No
Patient 2	Day 1	Buccal	Pre-treatment	Yes	1	Yes	AB Category 0	No	0	No	No	No	No	Grade 0	No	No	No	Mild	Yes	0	No
Patient 2	Day 3	Buccal	Treatment 1	No	0	No	AB Category 0	No	0	No	No	No	No	Grade 0	No	No	No	Mild	Yes	5	Yes
Patient 2	Day 8	Buccal	Treatment 1	Yes	1	Yes	AB Category 0	No	0	No	No	No	No	Grade 0	No	No	No	No	No	0	No
Patient 2	Day 11	Buccal	Treatment 1	Yes	4	Yes	AB Category 0	No	0	No	No	No	No	Grade 0	No	Severe	Yes	No	No	0	No
Patient 2	Day 14	Buccal	Treatment 1	Yes	3	Yes	AB Category 0	No	0	No	No	No	Yes	Grade 1	Yes	Severe	Yes	No	No	5	Yes
Patient 2	Day 17	Buccal	Treatment 1	No	0	No	AB Category 3	Yes	6	Yes	No	Yes	Yes	Grade 2	Yes	Severe	Yes	Severe	Yes	0	No
Patient 2	Day 24	Buccal	Treatment 1	No	0	No	AB Category 0	No	26	Yes	Yes	No	Yes	Grade 1	Yes	Moderate	Yes	No	No	0	No
Patient 2	Day 28	Buccal	Treatment 2	Yes	1	Yes	AB Category 0	No	10	Yes	No	No	No	Grade 0	No	Mild	Yes	No	No	0	No
Patient 2	Day 32	Buccal	Treatment 2	Yes	3	Yes	AB Category 0	No	0	No	No	No	No	Grade 0	No	No	No	No	No	0	No
Patient 2	Day 39	Buccal	Treatment 2	No	0	No	AB Category 2	Yes	2	Yes	Yes	No	No	Grade 0	No	Severe	Yes	Mild	Yes	0	No
Patient 2	Day 50	Buccal	Treatment 3	Yes	2	Yes	AB Category 0	No	0	No	Yes	No	No	Grade 0	No	Mild	Yes	No	No	0	No
Patient 2	Day 56	Buccal	Treatment 3	Yes	8	Yes	AB Category 0	No	1	Yes	Yes	No	No	Grade 0	No	Mild	Yes	Mild	Yes	0	No
Patient 2	Day 198	Buccal	Follow-up	No	0	No	AB Category 0	No	0	No	No	No	No	Grade 0	No	No	No	No	No	0	No
Patient 2	Day 14	Lesion	Treatment 1	Yes	3	Yes	AB Category 0	No	0	No	No	No	Yes	Grade 1	Yes	Severe	Yes	No	No	5	Yes
Patient 2	Day 17	Lesion	Treatment 1	No	0	No	AB Category 3	Yes	6	Yes	No	Yes	Yes	Grade 2	Yes	Severe	Yes	Severe	Yes	0	No
Patient 2	Day 24	Lesion	Treatment 1	No	0	No	AB Category 0	No	26	Yes	Yes	No	Yes	Grade 1	Yes	Moderate	Yes	No	No	0	No

Patient	Time	Sample type	Sampling period	Chemo	Chemo load	Chemo load Y/N	Antibiotic category	AB Y/N	AB load	AB load Y/N	AB rinse	Antimycotic rinse	LLLT	Mucositis grade	Mucositis Y/N	Neutropenia grade	Neutropenia Y/N	Inflammation grade	Inflammation Y/N	Pain score	Pain Y/N
Patient 2	Day 28	Lesion	Treatment 2	Yes	1	Yes	AB Category 0	No	10	Yes	No	No	No	Grade 0	No	Mild	Yes	No	No	0	No
Patient 3	Day 5	Saliva	Treatment 1	No	0	No	AB Category 0	No	0	No	No	No	No	Grade 0	No	No	No	No	No	0	No
Patient 3	Day 9	Saliva	Treatment 1	Yes	2	Yes	AB Category 0	No	0	No	No	No	No	Grade 0	No	No	No	No	No	0	No
Patient 3	Day 16	Saliva	Treatment 1	No	0	No	AB Category 0	No	0	No	No	No	No	Grade 0	No	Mild	Yes	No	No	0	No
Patient 3	Day 20	Saliva	Treatment 1	No	0	No	AB Category 0	No	0	No	Yes	Yes	Yes	Grade 2	Yes	Severe	Yes	No	No	0	No
Patient 3	Day 23	Saliva	Treatment 1	No	0	No	AB Category 3	Yes	9	Yes	Yes	Yes	Yes	Grade 1	Yes	Severe	Yes	Mild	Yes	0	No
Patient 3	Day 29	Saliva	Treatment 2	Yes	1	Yes	AB Category 0	No	0	No	No	No	No	Grade 0	No	No	No	No	No	0	No
Patient 3	Day 34	Saliva	Treatment 2	Yes	4	Yes	AB Category 0	No	0	No	No	No	No	Grade 0	No	No	No	No	No	0	No
Patient 3	Day 40	Saliva	Treatment 2	No	0	No	AB Category 0	No	1	Yes	Yes	No	No	Grade 2	Yes	Severe	Yes	Mild	Yes	4	Yes
Patient 3	Day 47	Saliva	Treatment 2	No	0	No	AB Category 0	No	1	Yes	Yes	No	No	Grade 1	Yes	No	No	Mild	Yes	0	No
Patient 3	Day 51	Saliva	Treatment 3	Yes	2	Yes	AB Category 0	No	0	No	No	No	No	Grade 2	Yes	Mild	Yes	No	No	0	No
Patient 3	Day 55	Saliva	Treatment 3	Yes	6	Yes	AB Category 0	No	0	No	No	No	No	Grade 1	Yes	Mild	Yes	No	No	0	No
Patient 3	Day 61	Saliva	Treatment 3	No	2	Yes	AB Category 0	No	1	Yes	Yes	No	No	Grade 0	No	Severe	Yes	No	No	0	No
Patient 3	Day 75	Saliva	Treatment 3	No	0	No	AB Category 0	No	1	Yes	Yes	No	No	Grade 0	No	Mild	Yes	No	No	0	No
Patient 3	Day 146	Saliva	Follow-up	No	0	No	AB Category 0	No	0	No	No	No	No	Grade 0	No	No	No	No	No	0	No
Patient 3	Day 5	Buccal	Treatment 1	No	0	No	AB Category 0	No	0	No	No	No	No	Grade 0	No	No	No	No	No	0	No
Patient 3	Day 9	Buccal	Treatment 1	Yes	2	Yes	AB Category 0	No	0	No	No	No	No	Grade 0	No	No	No	No	No	0	No
Patient 3	Day 16	Buccal	Treatment 1	No	0	No	AB Category 0	No	0	No	No	No	No	Grade 0	No	Mild	Yes	No	No	0	No
Patient 3	Day 20	Buccal	Treatment 1	No	0	No	AB Category 0	No	0	No	Yes	Yes	Yes	Grade 2	Yes	Severe	Yes	No	No	0	No
Patient 3	Day 23	Buccal	Treatment 1	No	0	No	AB Category 3	Yes	9	Yes	Yes	Yes	Yes	Grade 1	Yes	Severe	Yes	Mild	Yes	0	No
Patient 3	Day 29	Buccal	Treatment 2	Yes	1	Yes	AB Category 0	No	0	No	No	No	No	Grade 0	No	No	No	No	No	0	No
Patient 3	Day 34	Buccal	Treatment 2	Yes	4	Yes	AB Category 0	No	0	No	No	No	No	Grade 0	No	No	No	No	No	0	No
Patient 3	Day 40	Buccal	Treatment 2	No	0	No	AB Category 0	No	1	Yes	Yes	No	No	Grade 2	Yes	Severe	Yes	Mild	Yes	4	Yes
Patient 3	Day 47	Buccal	Treatment 2	No	0	No	AB Category 0	No	1	Yes	Yes	No	No	Grade 1	Yes	No	No	Mild	Yes	0	No
Patient 3	Day 51	Buccal	Treatment 3	Yes	2	Yes	AB Category 0	No	0	No	No	No	No	Grade 2	Yes	Mild	Yes	No	No	0	No
Patient 3	Day 55	Buccal	Treatment 3	Yes	6	Yes	AB Category 0	No	0	No	No	No	No	Grade 1	Yes	Mild	Yes	No	No	0	No

Patient	Time	Sample type	Sampling period	Chemo	Chemo load	Chemo load Y/N	Antibiotic category	AB Y/N	AB load	AB load Y/N	AB rinse	Antimycotic rinse	LLLT	Mucositis grade	Mucositis Y/N	Neutropenia grade	Neutropenia Y/N	Inflammation grade	Inflammation Y/N	Pain score	Pain Y/N
Patient 3	Day 61	Buccal	Treatment 3	No	2	Yes	AB Category 0	No	1	Yes	Yes	No	No	Grade 0	No	Severe	Yes	No	No	0	No
Patient 3	Day 75	Buccal	Treatment 3	No	0	No	AB Category 0	No	1	Yes	Yes	No	No	Grade 0	No	Mild	Yes	No	No	0	No
Patient 3	Day 146	Buccal	Follow-up	No	0	No	AB Category 0	No	0	No	No	No	No	Grade 0	No	No	No	No	No	0	No
Patient 3	Day 20	Lesion	Treatment 1	No	0	No	AB Category 0	No	0	No	Yes	Yes	Yes	Grade 2	Yes	Severe	Yes	No	No	0	No
Patient 3	Day 23	Lesion	Treatment 1	No	0	No	AB Category 3	Yes	9	Yes	Yes	Yes	Yes	Grade 1	Yes	Severe	Yes	Mild	Yes	0	No
Patient 3	Day 40	Lesion	Treatment 2	No	0	No	AB Category 0	No	1	Yes	Yes	No	No	Grade 2	Yes	Severe	Yes	Mild	Yes	4	Yes
Patient 3	Day 47	Lesion	Treatment 2	No	0	No	AB Category 0	No	1	Yes	Yes	No	No	Grade 1	Yes	No	No	Mild	Yes	0	No
Patient 3	Day 55	Lesion	Treatment 3	Yes	6	Yes	AB Category 0	No	0	No	No	No	No	Grade 1	Yes	Mild	Yes	No	No	0	No
Patient 4	Day 13	Saliva	Treatment 1	Yes	9	Yes	AB Category 0	No	0	No	No	Yes	No	Grade 1	Yes	Severe	Yes	No	No	1	Yes
Patient 4	Day 16	Saliva	Treatment 1	No	6	Yes	AB Category 3	Yes	9	Yes	No	Yes	Yes	Grade 2	Yes	Severe	Yes	Mild	Yes	2	Yes
Patient 4	Day 20	Saliva	Treatment 1	No	2	Yes	AB Category 4	Yes	23	Yes	No	Yes	Yes	Grade 3	Yes	Severe	Yes	Moderate	Yes	2	Yes
Patient 4	Day 27	Saliva	Treatment 1	No	0	No	AB Category 4	Yes	51	Yes	No	Yes	Yes	Grade 2	Yes	No	No	Mild	Yes	1	Yes
Patient 4	Day 30	Saliva	Treatment 2	Yes	1	Yes	AB Category 0	No	39	Yes	No	No	No	Grade 0	No	No	No	Mild	Yes	0	No
Patient 4	Day 34	Saliva	Treatment 2	Yes	3	Yes	AB Category 0	No	23	Yes	No	No	No	Grade 0	No	No	No	Mild	Yes	0	No
Patient 4	Day 37	Saliva	Treatment 2	Yes	6	Yes	AB Category 0	No	11	Yes	No	No	No	Grade 0	No	No	No	No	No	2	Yes
Patient 4	Day 41	Saliva	Treatment 2	No	4	Yes	AB Category 2	Yes	19	Yes	No	No	Yes	Grade 1	Yes	Severe	Yes	No	No	2	Yes
Patient 4	Day 44	Saliva	Treatment 2	No	1	Yes	AB Category 2	Yes	25	Yes	No	No	Yes	Grade 3	Yes	Severe	Yes	Moderate	Yes	4	Yes
Patient 4	Day 47	Saliva	Treatment 2	No	0	No	AB Category 4	Yes	35	Yes	No	No	Yes	Grade 2	Yes	Severe	Yes	Moderate	Yes	3	Yes
Patient 4	Day 51	Saliva	Treatment 2	No	0	No	AB Category 4	Yes	51	Yes	No	No	Yes	Grade 1	Yes	Severe	Yes	Moderate	Yes	2	Yes
Patient 4	Day 63	Saliva	Treatment 2	No	0	No	AB Category 0	No	61	Yes	No	No	No	Grade 0	No	Mild	Yes	No	No	0	No
Patient 4	Day 72	Saliva	Treatment 2	No	0	No	AB Category 0	No	27	Yes	No	No	No	Grade 0	No	Mild	Yes	No	No	0	No
Patient 4	Day 226	Saliva	Follow-up	No	0	No	AB Category 0	No	0	No	No	No	No	Grade 0	No	Mild	Yes	No	No	0	No
Patient 4	Day 13	Buccal	Treatment 1	Yes	9	Yes	AB Category 0	No	0	No	No	Yes	No	Grade 1	Yes	Severe	Yes	No	No	1	Yes
Patient 4	Day 16	Buccal	Treatment 1	No	6	Yes	AB Category 3	Yes	9	Yes	No	Yes	Yes	Grade 2	Yes	Severe	Yes	Mild	Yes	2	Yes
Patient 4	Day 20	Buccal	Treatment 1	No	2	Yes	AB Category 4	Yes	23	Yes	No	Yes	Yes	Grade 3	Yes	Severe	Yes	Moderate	Yes	2	Yes
Patient 4	Day 27	Buccal	Treatment 1	No	0	No	AB Category 4	Yes	51	Yes	No	Yes	Yes	Grade 2	Yes	No	No	Mild	Yes	1	Yes

Patient	Time	Sample type	Sampling period	Chemo	Chemo load	Chemo load Y/N	Antibiotic category	AB Y/N	AB load	AB load Y/N	AB rinse	Antimycotic rinse	LLLT	Mucositis grade	Mucositis Y/N	Neutropenia grade	Neutropenia Y/N	Inflammation grade	Inflammation Y/N	Pain score	Pain Y/N
Patient 4	Day 30	Buccal	Treatment 2	Yes	1	Yes	AB Category 0	No	39	Yes	No	No	No	Grade 0	No	No	No	Mild	Yes	0	No
Patient 4	Day 34	Buccal	Treatment 2	Yes	3	Yes	AB Category 0	No	23	Yes	No	No	No	Grade 0	No	No	No	Mild	Yes	0	No
Patient 4	Day 37	Buccal	Treatment 2	Yes	6	Yes	AB Category 0	No	11	Yes	No	No	No	Grade 0	No	No	No	No	No	2	Yes
Patient 4	Day 41	Buccal	Treatment 2	No	4	Yes	AB Category 2	Yes	19	Yes	No	No	Yes	Grade 1	Yes	Severe	Yes	No	No	2	Yes
Patient 4	Day 44	Buccal	Treatment 2	No	1	Yes	AB Category 2	Yes	25	Yes	No	No	Yes	Grade 3	Yes	Severe	Yes	Moderate	Yes	4	Yes
Patient 4	Day 47	Buccal	Treatment 2	No	0	No	AB Category 4	Yes	35	Yes	No	No	Yes	Grade 2	Yes	Severe	Yes	Moderate	Yes	3	Yes
Patient 4	Day 51	Buccal	Treatment 2	No	0	No	AB Category 4	Yes	51	Yes	No	No	Yes	Grade 1	Yes	Severe	Yes	Moderate	Yes	2	Yes
Patient 4	Day 63	Buccal	Treatment 2	No	0	No	AB Category 0	No	61	Yes	No	No	No	Grade 0	No	Mild	Yes	No	No	0	No
Patient 4	Day 72	Buccal	Treatment 2	No	0	No	AB Category 0	No	27	Yes	No	No	No	Grade 0	No	Mild	Yes	No	No	0	No
Patient 4	Day 226	Buccal	Follow-up	No	0	No	AB Category 0	No	0	No	No	No	No	Grade 0	No	Mild	Yes	No	No	0	No
Patient 4	Day 16	Lesion	Treatment 1	No	6	Yes	AB Category 3	Yes	9	Yes	No	Yes	Yes	Grade 2	Yes	Severe	Yes	Mild	Yes	2	Yes
Patient 4	Day 20	Lesion	Treatment 1	No	2	Yes	AB Category 4	Yes	23	Yes	No	Yes	Yes	Grade 3	Yes	Severe	Yes	Moderate	Yes	2	Yes
Patient 4	Day 27	Lesion	Treatment 1	No	0	No	AB Category 4	Yes	51	Yes	No	Yes	Yes	Grade 2	Yes	No	No	Mild	Yes	1	Yes
Patient 4	Day 41	Lesion	Treatment 2	No	4	Yes	AB Category 2	Yes	19	Yes	No	No	Yes	Grade 1	Yes	Severe	Yes	No	No	2	Yes
Patient 4	Day 44	Lesion	Treatment 2	No	1	Yes	AB Category 2	Yes	25	Yes	No	No	Yes	Grade 3	Yes	Severe	Yes	Moderate	Yes	4	Yes
Patient 4	Day 47	Lesion	Treatment 2	No	0	No	AB Category 4	Yes	35	Yes	No	No	Yes	Grade 2	Yes	Severe	Yes	Moderate	Yes	3	Yes
Patient 4	Day 51	Lesion	Treatment 2	No	0	No	AB Category 4	Yes	51	Yes	No	No	Yes	Grade 1	Yes	Severe	Yes	Moderate	Yes	2	Yes
Patient 5	Day 2	Saliva	Pre-treatment	Yes	2	Yes	AB Category 0	No	0	No	No	No	No	Grade 0	No	Severe	Yes	No	No		No
Patient 5	Day 6	Saliva	Treatment 1	Yes	6	Yes	AB Category 0	No	0	No	No	No	No	Grade 0	No	Severe	Yes	No	No	0	No
Patient 5	Day 9	Saliva	Treatment 1	Yes	9	Yes	AB Category 3	Yes	6	Yes	No	Yes	Yes	Grade 0	No	Severe	Yes	Moderate	Yes	5	Yes
Patient 5	Day 15	Saliva	Treatment 1	No	9	Yes	AB Category 3	Yes	24	Yes	No	Yes	No	Grade 1	Yes	Severe	Yes	Mild	Yes	0	No
Patient 5	Day 19	Saliva	Treatment 1	No	5	Yes	AB Category 2	Yes	32	Yes	Yes	No	No	Grade 1	Yes	Severe	Yes	Mild	Yes	0	No
Patient 5	Day 26	Saliva	Treatment 1	No	0	No	AB Category 4	Yes	54	Yes	Yes	No	No	Grade 2	Yes	Severe	Yes	Mild	Yes	0	No
Patient 5	Day 34	Saliva	Treatment 1	No	0	No	AB Category 2	Yes	84	Yes	Yes	No	No	Grade 0	No	Severe	Yes	No	No	0	No
Patient 5	Day 42	Saliva	Treatment 2	Yes	2	Yes	AB Category 2	Yes	100	Yes	Yes	No	No	Grade 0	No	Mild	Yes	No	No	0	No
Patient 5	Day 47	Saliva	Treatment 2	Yes	7	Yes	AB Category 2	Yes	110	Yes	Yes	No	No	Grade 0	No	Mild	Yes	No	No	0	No

Patient	Time	Sample type	Sampling period	Chemo	Chemo load	Chemo load Y/N	Antibiotic category	AB Y/N	AB load	AB load Y/N	AB rinse	Antimycotic rinse	LLLT	Mucositis grade	Mucositis Y/N	Neutropenia grade	Neutropenia Y/N	Inflammation grade	Inflammation Y/N	Pain score	Pain Y/N
Patient 5	Day 56	Saliva	Treatment 2	No	0	No	AB Category 4	Yes	133	Yes	Yes	Yes	Yes	Grade 2	Yes	Severe	Yes	Moderate	Yes	6	Yes
Patient 5	Day 58	Saliva	Treatment 2	No	0	No	AB Category 4	Yes	141	Yes	Yes	Yes	Yes	Grade 3	Yes	Severe	Yes	Moderate	Yes	5	Yes
Patient 5	Day 62	Saliva	Treatment 2	No	0	No	AB Category 4	Yes	157	Yes	Yes	No	No	Grade 3	Yes	Severe	Yes	Moderate	Yes	2	Yes
Patient 5	Day 68	Saliva	Treatment 2	No	0	No	AB Category 4	Yes	181	Yes	Yes	No	No	Grade 2	Yes	Severe	Yes	Severe	Yes	0	No
Patient 5	Day 86	Saliva	Treatment 2	No	0	No	AB Category 3	Yes	253	Yes	Yes	No	No	Grade 0	No	Mild	Yes	No	No	0	No
Patient 5	Day 2	Buccal	Pre-treatment	Yes	2	Yes	AB Category 0	No	0	No	No	No	No	Grade 0	No	Severe	Yes	No	No		No
Patient 5	Day 6	Buccal	Treatment 1	Yes	6	Yes	AB Category 0	No	0	No	No	No	No	Grade 0	No	Severe	Yes	No	No	0	No
Patient 5	Day 9	Buccal	Treatment 1	Yes	9	Yes	AB Category 3	Yes	6	Yes	No	Yes	Yes	Grade 0	No	Severe	Yes	Moderate	Yes	5	Yes
Patient 5	Day 15	Buccal	Treatment 1	No	9	Yes	AB Category 3	Yes	24	Yes	No	Yes	No	Grade 1	Yes	Severe	Yes	Mild	Yes	0	No
Patient 5	Day 19	Buccal	Treatment 1	No	5	Yes	AB Category 2	Yes	32	Yes	Yes	No	No	Grade 1	Yes	Severe	Yes	Mild	Yes	0	No
Patient 5	Day 26	Buccal	Treatment 1	No	0	No	AB Category 4	Yes	54	Yes	Yes	No	No	Grade 2	Yes	Severe	Yes	Mild	Yes	0	No
Patient 5	Day 34	Buccal	Treatment 1	No	0	No	AB Category 2	Yes	84	Yes	Yes	No	No	Grade 0	No	Severe	Yes	No	No	0	No
Patient 5	Day 42	Buccal	Treatment 2	Yes	2	Yes	AB Category 2	Yes	100	Yes	Yes	No	No	Grade 0	No	Mild	Yes	No	No	0	No
Patient 5	Day 47	Buccal	Treatment 2	Yes	7	Yes	AB Category 2	Yes	110	Yes	Yes	No	No	Grade 0	No	Mild	Yes	No	No	0	No
Patient 5	Day 56	Buccal	Treatment 2	No	0	No	AB Category 4	Yes	133	Yes	Yes	Yes	Yes	Grade 2	Yes	Severe	Yes	Moderate	Yes	6	Yes
Patient 5	Day 58	Buccal	Treatment 2	No	0	No	AB Category 4	Yes	141	Yes	Yes	Yes	Yes	Grade 3	Yes	Severe	Yes	Moderate	Yes	5	Yes
Patient 5	Day 62	Buccal	Treatment 2	No	0	No	AB Category 4	Yes	157	Yes	Yes	No	No	Grade 3	Yes	Severe	Yes	Moderate	Yes	2	Yes
Patient 5	Day 68	Buccal	Treatment 2	No	0	No	AB Category 4	Yes	181	Yes	Yes	No	No	Grade 2	Yes	Severe	Yes	Severe	Yes	0	No
Patient 5	Day 86	Buccal	Treatment 2	No	0	No	AB Category 3	Yes	253	Yes	Yes	No	No	Grade 0	No	Mild	Yes	No	No	0	No
Patient 5	Day 56	Lesion	Treatment 2	No	0	No	AB Category 4	Yes	133	Yes	Yes	Yes	Yes	Grade 2	Yes	Severe	Yes	Moderate	Yes	6	Yes
Patient 5	Day 58	Lesion	Treatment 2	No	0	No	AB Category 4	Yes	141	Yes	Yes	Yes	Yes	Grade 3	Yes	Severe	Yes	Moderate	Yes	5	Yes
Patient 5	Day 62	Lesion	Treatment 2	No	0	No	AB Category 4	Yes	157	Yes	Yes	No	No	Grade 3	Yes	Severe	Yes	Moderate	Yes	2	Yes
Patient 5	Day 68	Lesion	Treatment 2	No	0	No	AB Category 4	Yes	181	Yes	Yes	No	No	Grade 2	Yes	Severe	Yes	Severe	Yes	0	No

CHAPTER 6

General discussion

CHAPTER 6
General discussion

1. Positioning of the research

Although chemotherapeutic agents for cancer treatment already exist for more than 75 years and supportive care is getting more and more attention, cancer patients still have to deal with many side effects of chemotherapy. One of the major side effects is **mucositis**, an inflammation and ulceration of the mucosa, which can occur along the entire alimentary tract. It has a large negative impact on the quality of life of patients and can cause delay of the cancer treatment. Unfortunately, treatment options are elusive. Many chemotherapeutic agents are known to induce mucositis. In this thesis, we chose to focus on 5-FU and irinotecan (SN-38), two commonly used chemotherapeutic agents with high incidences of mucositis.

In line with the general increasing interest in host **microbiome** research, the microbiota are thought to play an essential role in the development of both oral and gastrointestinal mucositis. Host-microbe interaction processes during mucositis and the impact of chemotherapy on these interactions are however largely underexplored. Certain studies showed that chemotherapy induces clear shifts in the microbiome, yet shifts are inconsistent between studies. The basic research question that prevails concerns the etiology of mucositis: 1) do chemotherapeutics directly cause microbial dysbiosis, thereby increasing mucositis risk or 2) does the chemotherapy subjected host environment disturb the microbiome thereby aggravating the disease.

In higher grades of mucositis, **ulcerations** will increase the pain and discomfort for the patient. These ulcers are highly colonized by bacteria and the loss of mucosal barrier will increase the risk of infection. The dysbiotic state of the microbiome may further provide the opportunity to pathogens to cause both local and systemic infections. **Healing** of the wounds is therefore essential for the recovery from mucositis and usually occurs spontaneously. However, the impact of the bacteria during the healing of these wounds is largely unknown.

To further unravel the role of the host microbiota in the development of chemotherapy-induced mucositis, we investigated 1) the direct effect of chemotherapeutic agents on both oral and gastrointestinal microbiota *in vitro*, 2) the interactions between the host, its microbiota and chemotherapeutic treatment both *in vitro* and *in vivo*.

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2. Main research outcomes

To gain more knowledge about the impact of chemotherapy on the microbiota in the context of alimentary mucositis different experimental set-ups were applied: a monoculture study (Chapter 2), an *in vitro* colon simulation (Chapter 3), an *in vitro* oral mucosa model (Chapter 4), and an *in vivo* longitudinal observational study of the oral microbiome (Chapter 5). The main research outcomes are summarized in Figure 6.1.

In **Chapter 2** a monoculture study showed that 5-FU sensitivity varies among oral microorganisms at physiologically relevant concentrations. Some species were highly resistant to all tested concentrations (0.1-50 µM 5-FU), whereas others had a reduced growth and viability already at 0.4 µM 5-FU. Dihydropyrimidine dehydrogenase, an enzyme involved in 5-FU resistance in humans, did not impact the resistance of the microorganisms.

An ecosystemic approach was used in **Chapter 3** to assess the effect of chemotherapy on the gut microbiome. Fecal samples of different healthy individuals were incubated in an M-SHIME® (Mucosal-Simulator of the Human Intestinal Ecosystem) and exposed to 5-FU or SN-38 (active metabolite of irinotecan). For both chemotherapeutic agents, no clear effect on metabolic activity or microbial composition was observed. As this *in vitro* model does not include a host compartment, we hypothesize that the host is crucial in the establishment of chemotherapy-induced microbial shifts *in vivo*.

Based on these findings, the second part of this research focused on the interactions between microbiota, chemotherapy and the host. The research in **Chapter 4** showed that oral microbiota reduce wound healing capacity of oral epithelial cells, an important process in recovering from mucositis. For healthy donors, wound healing capacity was inversely correlated with microbial load. However, this was not the case for patient samples, probably due to the higher abundance of pathogenic genera combined with a less diverse microbial community. Again, the direct impact of 5-FU on the microbiome was limited.

Finally, an *in vivo* longitudinal study of the oral microbiome of pediatric patients treated for hematological malignancies and suffering from oral mucositis was performed in **Chapter 5**. Major patient-specific shifts in the oral microbial community were observed during and following chemotherapeutic treatments. Chemotherapy and mucositis only induced minor microbial shifts, whereas the use of antibiotics had a major impact. Also neutropenia, inflammation and the use of antibacterial mouth rinse with chlorhexidine correlated with microbial changes. Despite the major shifts, the microbial community partially recovered after therapy.

Overall, this research provided unique and valuable information concerning the effect of chemotherapy on the host microbiota. However, inclusion of more individuals will be crucial in future research to cover biological and interindividual variability.

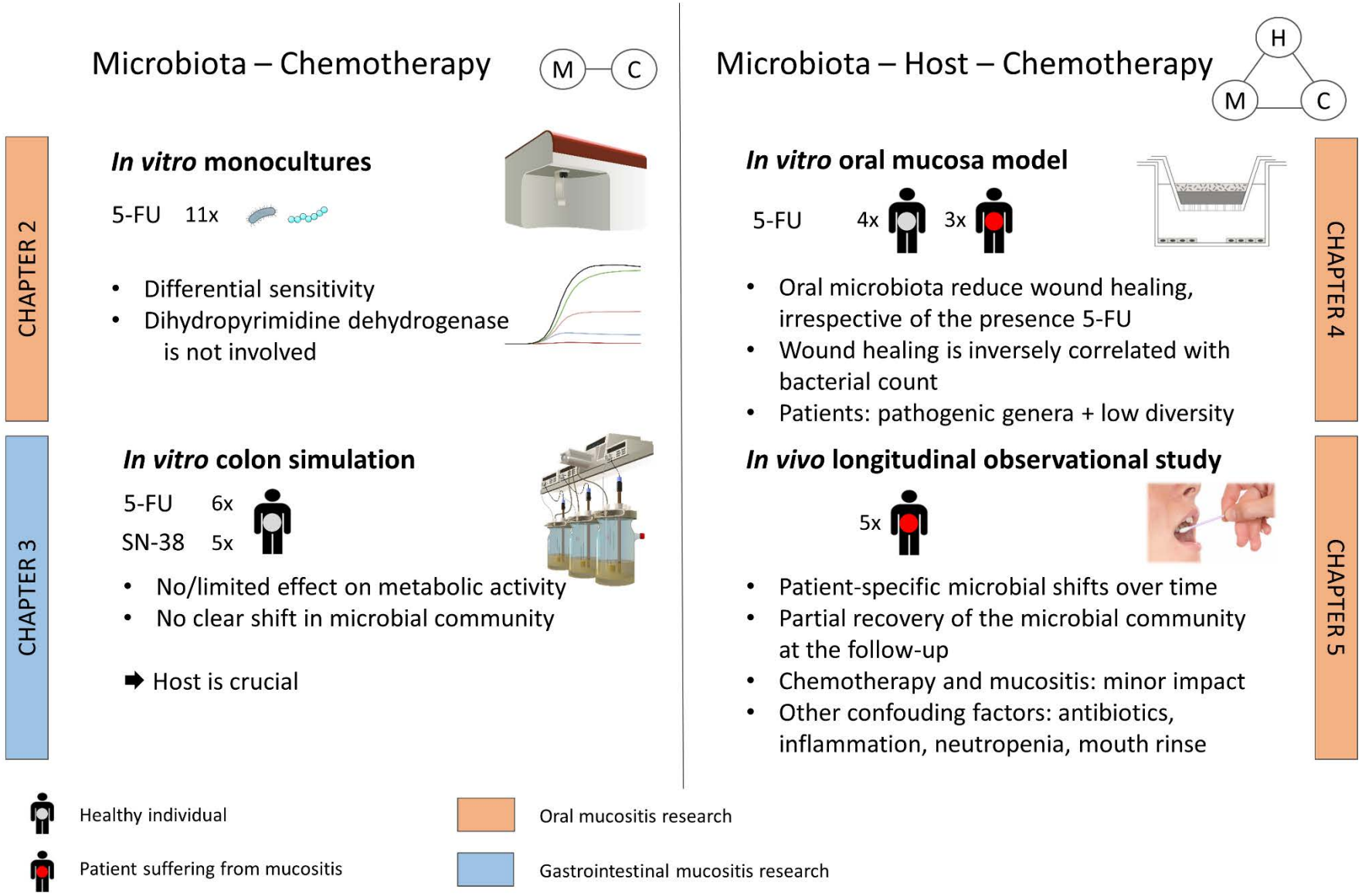

Figure 6.1 - Schematic overview of the research chapters and their main outcomes.

3. Crucial factors in chemotherapy-induced microbial shifts

3.1 The importance of the host and its immune system in chemotherapy-induced mucositis

Pre-clinical models and clinical studies have already reported changes in the microbiome during both oral (reviewed by Vanhoecke et al. 2015b, Vasconcelos et al. 2016) and gastrointestinal (reviewed by Stringer 2013, Touchefeu et al. 2014) mucositis, which are described in detail in Chapter 1. Our *in vitro* research however, showed a limited direct effect of chemotherapeutic agents on the host microbiome (Chapter 3 and 4). As our models did not comprise a host (immune system) compartment, this indicates that the **host and its immune system** are crucial factors mediating the chemotherapy-induced microbial changes. Hence, we hypothesize that the chemotherapy-disturbed host environment and not the direct effect of the chemotherapy on the microbiome, is the primary trigger of microbial changes, thereby aggravating the mucositis process.

Following chemotherapy, an **inflammatory status** with high levels of pro-inflammatory cytokines and NF-κB characterizes the mucosa during the first phases of mucositis (Sonis 2004, Logan et al. 2008a). These host-related changes of the environment may impact the microbiome. Once the inflammatory state is established by the chemotherapeutic agent, microbiota can further aggravate or reduce the inflammation, depending on the microbial community composition. Some commensals can reduce active NF-κB levels (Kelly et al. 2004, O'hara et al. 2006), whereas pathogens can increase the inflammation by activation of TLR/NF-κB pathway via MAMPs (Vanhoecke and Stringer 2015, Vasconcelos et al. 2016).

In Chapter 1, a **triangular model** was proposed to describe the interactions between chemotherapy, the host and its microbiota. However, our results show that the impact of chemotherapeutic agents on the microbiota is limited. Therefore, we propose a more **linear model**, in which the effect of chemotherapy to the microbiome is expressed via the host (Figure 6.2). In this model, the host environment will first be disturbed by the chemotherapeutic agent which induces an inflammatory status. This will induce microbial changes, which may further aggravate the mucositis process.

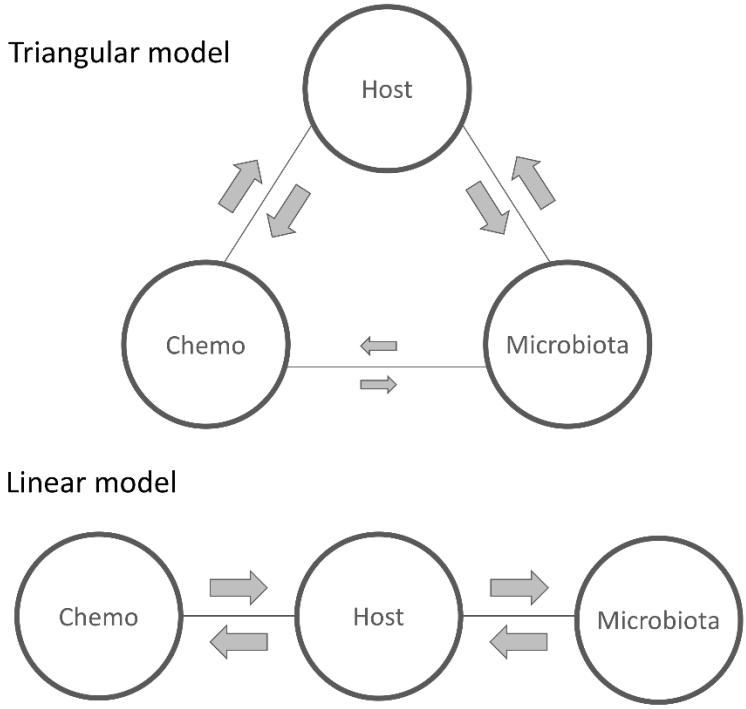

Figure 6.2 – Interactions between host, microbiota and chemotherapy: from a triangular to a linear model.

3.2 The importance of antibiotics in chemotherapy-induced mucositis

Next to the host-mediated effects of the chemotherapeutic agents, other factors can impact the microbiome during cancer treatment and mucositis. An important factor, which has been largely discussed in literature, is the use of antibiotics. Different **local antimicrobial agents** were tested to reduce oral mucositis. However, none of them is recommended by the MASCC/ISOO guidelines (Lalla et al. 2014). Multiple studies showed that a local oral antibiotic treatment with polymyxin E, tobramycin and amphotericin B (PTA) did not impact the incidence or severity of oral mucositis, although it did change the microbial community composition by lowering the abundance of Gram-negative species (Wijers et al. 2001, Stokman et al. 2003, Stokman et al. 2006). The antibacterial agent chlorhexidine is still commonly used to prevent mucositis or to reduce its severity (Rodriguez-Caballero et al. 2012). As conflicting results were reported (Stokman et al. 2006, Stringer and Logan 2015), no guideline towards its use in patients treated with chemotherapy could be set (Lalla et al. 2014). For patients receiving radiotherapy however, the use of chlorhexidine is not recommended (Lalla et al. 2014). Recently, a meta-analysis further discouraged the use of chlorhexidine during mucositis as no positive effect on mucositis was reported (Cardona et al. 2017). Compared to brushing the teeth, chlorhexidine use did not have an inhibitory effect towards mucositis development, nor towards infection risk (Antunes et al. 2010a). The main goal of all these local antimicrobials is to selectively eliminate certain oral microbiota, such as aerobic Gram-negative bacteria (Wijers et al. 2001). Thereby, these antimicrobials all lead to shifts in the microbiome. In our *in vivo*

study, we indeed observed an impact of chlorhexidine mouth rinse on the oral microbial community composition (Chapter 5). Similarly, the use of chlorhexidine mouth rinse by healthy individuals, shifted their tongue and saliva microbial community composition (Exterkate et al. 2015). *In vitro*, chlorhexidine has been shown to reduce the relative abundance of *Streptococcus*, *Solobacterium* and *Megasphaera,* the microbial diversity and the amount of viable cells of oral biofilms (Mostajo et al. 2017). This all indicates a shift to a disturbed microbial community and a lack of clinical evidence in favor of the use of chlorhexidine for mucositis. Therefore, we do not support the use of a chlorhexidine mouth rinse in mucositis patients, which is in line with the MASCC/ISOO guidelines (Lalla et al. 2014).

Not only local antimicrobial agents, but also **systemic antibiotics** may have large effects on the host microbiome (Chapter 5). Both therapeutic and prophylactic antibiotics can impact all host microbiota with disturbances depending on the specific agent, the absorption, the route of elimination, and possible enzymatic inactivation and/or binding to fecal material (Sullivan et al. 2001). Most of the research concerning the impact of systemic antibiotics on the host microbiome is conducted focusing on the gastrointestinal microbiome in the context of antibiotic-associated diarrhea, a frequent side effect of antibiotic treatment (Sullivan et al. 2001, Silverman et al. 2017). However, also the oral microbiome can be disturbed by systemic antibiotics (Sullivan et al. 2001), although more research is needed to get a better view on the impact of systemic antibiotics towards the oral microbiome. The use of antibiotics may not only shift the commensal microbiome, but also change the infection pattern. The use of prophylactic antibiotics for high risk cancer patients was previously reported to cause a prominent shift in infection cultures from Gram-negative to Gram-positive species in the nineties (Zinner 1999). This leads to large numbers of *Streptococcus viridans* infections in cancer patients (section 4).

Thus, both local and systemic antibiotics may impact the oral and gut microbiome. The microbiota are however crucial in maintaining homeostasis in both the oral cavity (Marsh 2012) and the gastrointestinal tract (Sekirov et al. 2010). The establishment of colonization resistance by a normal healthy community, for example, will prevent the colonization of exogenous pathogens. However, long-term use of antibiotics will suppress the resident microbiota, leading to a **dysbiotic ecosystem** in which exogenous microbiota are able to colonize the oral cavity or the gastrointestinal tract (Marsh 2012, Silverman et al. 2017). Moreover, low abundant endogenous microbiota can become dominant and change the microbial composition and metabolic activity (Marsh 2003). The eradication of microbiota by use of prophylactic antibiotics resulted for example in an overgrowth of pathogens in the gut of AML patients (van Vliet et al. 2009). The common infectious pathogen *Clostridium difficile* is the most important source of antibiotic-associated diarrhea (Wilcox 2003). An unbalanced microbial ecosystem with lower microbial diversity and higher abundances of pathogenic species will probably be more sensitive to stress factors (Wittebolle et al. 2009). For one patient, lower microbial diversity

was indeed linked with major changes in the microbial community composition after chemotherapeutic treatment (Chapter 4). The interindividual response to chemotherapy for samples with a low diversity is probably related with a different microbial composition in combination with differential sensitivity to chemotherapy among microorganisms (Chapter 2).

In conclusion, chemotherapy will create a high inflammatory status which may induce changes in the microbiome. The use of systemic or local antibiotics may further disrupt the microbial community and thereby aggravate the mucositis process. Moreover, this unbalanced microbiome may be more susceptible to additional chemotherapeutic treatments, leading to an even more unbalanced microbiome.

4. Oral streptococci: friends or foes?

One of the most important bacterial genera in the oral cavity is ***Streptococcus***. All oral communities (saliva, tongue, plaque…) are highly colonized with streptococci, with the highest abundances (around 50 %) in the buccal mucosa (Segata et al. 2012). Their ability in adhering to teeth or mucosal surfaces makes them one of the pioneering species in biofilm formation. Cell multiplication and integration of other bacteria will create a mature biofilm and prevent the bacteria from being excreted with the salivary flow (Nobbs et al. 2009, Kolenbrander et al. 2010). Therefore, following each oral hygiene measure, streptococci are essential in rebuilding the oral biofilm (Kolenbrander et al. 2010). All our data confirmed that *Streptococcus* is a highly dominant genus in the oral cavity. In our *in vitro* co-culture model, biofilm formation based on oral samples was dominated with *Streptococcus* (Chapter 4). In the longitudinal patient study we observed high levels of *Streptococcus* in most of the samples and in general higher levels were seen in buccal samples, compared to saliva samples (Chapter 5). Moreover, all tested commensal *Streptococcus* strains were resistant to 5-FU in our study (Chapter 2). This indicates high resistance of streptococci against chemotherapeutic agents.

Although streptococci belong to the resident commensal oral microbiota, they often cause **infections** in cancer patients. In particular the **viridans streptococci** are known to be a major source of infection during neutropenia (Shenep 2000). These viridans streptococci are non-hemolytic or α-hemolytic and contain multiple species such as *S. mitis*, *S. oralis*, *S. sanguis*, *S. gordonii*, *S. crista*, *S. salivarius* and the *S. mutans* group. (Tunkel and Sepkowitz 2002). In the oral cavity, they are the first persistent colonizers after birth (Pearce et al. 1995). They not only reside in the oral cavity but also in the respiratory tract, the female genital tract and the entire gastrointestinal tract (Tunkel and Sepkowitz 2002). The attention for viridans streptococci infections has grown since the nineties, when the main strains causing blood stream infections in cancer patients with febrile neutropenia shifted from Gram-negative to Gram-positive species (Zinner 1999, Gustinetti and Mikulska 2016). However, today the

reverse trend is observed in many hospital care units with Gram-negative species taking over again (Gustinetti and Mikulska 2016). Still, viridans streptococci are one of the important Gram-positive blood cultures. This resulted in the experimental use of mouth rinses targeting Gram-positive species, for example containing vancomycin. The use of vancomycin mouth rinse was correlated with low viridans streptococci sepsis in a retrospective study of pediatric AML. However, the overall blood stream infections were not affected and infections with *Staphylococcus* species were high (Brunet et al. 2006). In our *in vivo* study, one patient used a vancomycin mouth spray and this was correlated with a reduced abundance of *Streptococcus* in the oral samples (Chapter 5). However, during this period, a positive blood culture for viridans streptococci was observed for this patient. Possible explanations are that the residing viridans streptococci became more pathogenic and virulent, or that the infection was coming from another source instead of the oral cavity. The stomach for example has been shown to be an important entry point for viridans streptococci following chemotherapeutic treatment (Tunkel and Sepkowitz 2002). Recent studies in our lab have shown that most of the oral microbiota (including multiple *Streptococcus* species) are much less sensitive to vancomycin, compared with chlorhexidine, at least for short incubation periods, mimicking the use of a mouth rinse (Beterams 2017). This all indicates that the use of vancomycin mouth spray is not advised to lower the infection risk, although more research is needed to confirm this.

In conclusion, the use of antibiotics will always favor certain microbiota, as the niche of the targeted microbiota becomes available for others. This may lead to an unbalanced community with low diversity and outgrowth of specific endogenous or exogenous pathogens. Therefore, the use of preventive antibiotics should be limited as much as possible to give the commensal microbiota the opportunity to re-establish their normal community, which will create homeostasis. During this re-establishment, streptococci will be crucial as they are the first colonizers of the immature biofilm. To reduce infection risk, it is however important to keep the absolute number of microbiota low for instance by mechanical cleaning such as tooth brushing.

5. (Future) use of *in vitro* models in mucositis research

5.1 *In vitro* versus *in vivo* studies in mucositis research

The impact of chemotherapy on the microbiome and the impact of the microbiome on mucositis can be studied both *in vitro* and *in vivo* each with their own strengths and limitations. While ***in vitro*** technologies will never exactly mimic the *in vivo* situation, physiological conditions are more accessible to high-throughput research and mechanistic studies in particular (Papadimitriou et al. 2015). Multi-parametric control allows several confounding factors to be controlled. This includes the use of microbiota from actual human origin as

opposed to animal microbiota from *in vivo* models and the possibility of including different types of host cells to study host-microbe interactions more in depth. In our case, *in vitro* setups for studying chemotherapy-microbiota interactions (Chapter 2 and 3) were used and models with the inclusion of host cells (Chapter 4) were used to study a specific phase of the mucositis process, namely wound healing. While our research primarily targeted epithelial processes, the addition of immune cells as a fourth element would make it even more physiologically relevant (Fontana et al. 2013).

In vivo research is always needed to confirm the mechanisms revealed by *in vitro* technologies. Both animal and clinical studies have the strength to be physiologically more relevant, but they are limited by higher costs and ethical constraints (Papadimitriou et al. 2015). Animal studies are largely used in mucositis research (Vanhoecke et al. 2015a). However, microbial shifts and host-microbe interactions might be different in animals in comparison to humans (Nguyen et al. 2015). Next to ethical constraints, clinical trials may have to deal with large numbers of confounding factors, that also impact the microbiome composition (Chapter 5). As our research revealed that the host and its immune system are crucial in chemotherapy-induced microbial shifts, the use of *in vivo* trials or *in vitro* technologies including host immune cells might be preferred in the future to get a better view on the host-microbiota-chemotherapy interactions.

5.2 (Future) opportunities for *in vitro* studies in mucositis research

Both in the *in vitro* co-culture model (Chapter 4) and in the *in vivo* observational study (Chapter 5), ***Streptococcus* and *Veillonella*** were highly abundant genera. *Streptococcus* is one of the initial biofilm colonizers (Kolenbrander et al. 2010) and is highly abundant in the buccal mucosa (Segata et al. 2012). Moreover, *Streptococcus* is acid-resistant and therefore important in caries, as it can convert glucose into lactic acid (Marsh 2003). *Veillonella* on the other hand, can consume this lactic acid and in this way take advantage of the presence of the streptococci (Kolenbrander 2000). So for healthy individuals, *Streptococcus* and *Veillonella* were the main genera included in the biofilm formation in the *in vitro* co-culture model.

In contrast, high interindividual differences in microbial composition were observed in the *in vitro* model when samples from patients suffering from mucositis were used. For each patient, one genus (*Streptococcus*, *Enterococcus* or *Abiotrophia*) was highly dominating the model, leading to a low diversity. As this mostly resembles *in vivo* mucositis lesion samples (Chapter 5) (Ye et al. 2013), this made us conclude that the oral *in vitro* co-culture model is primarily a **pathological model**, in which a certain genus/genera is/are dominating the community. The composition of the initial oral sample will determine the final composition in the model and has been shown to be more variable in patients suffering from mucositis compared to healthy individuals (Ye et al. 2013). Hence, by using saliva samples of patients,

a community more similar to the lesion microbiome is created in the model to investigate its interactions with the wound healing process.

In conclusion, the use of *in vitro* models is encouraged to further investigate the impact of microbiota on wound healing and to unravel the mechanisms behind. A longitudinal follow-up of patients samples in the model before, during and after mucositis, could provide more details on the effect of the shifting microbiome on wound healing capacity. Moreover, our studies only checked for shifts in the microbial DNA, based on 16S rRNA gene DGGE or Illumina amplicon sequencing. Measuring activity of the microbiome (RNA level), proteomics or metabolomics, could reveal more details about changes in activity profiles and underling mechanisms. Our data further suggest that to study chemotherapy-induced microbial shifts, the incorporation of a host compartment, including immune cells is essential in *in vitro* setups.

6. Treatment approaches for mucositis

6.1 Do oral and gastrointestinal mucositis need a different treatment approach?

Oral and gastrointestinal mucositis are both described by a 5-phases model comprising of an initiation, messenger amplification, proliferation, ulceration and healing phase (Sonis 2004). However, the question rises if this also implies that a similar treatment approach should be used for both pathologies? Several factors crucial for mucositis will be described below, to conclude with a suggested treatment approach.

Both oral and gastrointestinal mucositis are characterized by a disturbed **mucosal barrier** function. The combination of an increased epithelial permeability and the disruption of the mucus layer will enhance the ability of microbiota to reach the submucosa (Sonis 2004). This will make patients suffering from mucositis more prone to secondary infections, bacteremia and sepsis (Vasconcelos et al. 2016). In healthy conditions, the epithelial integrity is established by tight junction proteins, intercellular complexes providing a primary barrier. Alteration of tight junction proteins following chemotherapy can lead to ulcer development or reduced tissue integrity in both the gastrointestinal tract and in the oral cavity (Wardill et al. 2012, Wardill et al. 2016). Moreover, chemotherapy-induced diarrhea is linked with an increased intestinal permeability (Russo et al. 2013). Next to epithelial integrity, the mucosal barrier function is established by the mucus layer. Also here, chemotherapy can reduce this barrier compartment as it may impact mucus secretion (Stringer et al. 2009c).

An important feature in recovering from mucositis and in restoring the mucosal barrier is **wound healing**. A high **microbial load** is linked with a reduced wound healing capacity and increases the risk of infection (Chapter 4; Edwards and Harding 2004). The importance of microbial density in the wound healing process has previously been shown by De Ryck et al.

(2015). In other oral diseases such as gingivitis and periodontitis, a high microbial biomass is also linked with disease development (Meyle and Chapple 2015, Kilian et al. 2016). To reduce this microbial load in the oral cavity, good **oral hygiene** is essential, both in healthy and diseased state (McGuire et al. 2013). The use of oral care protocols, including regular basic oral hygiene (tooth brushing and flossing), professional examinations, education of patients and family and an interdisciplinary approach are advised by MASCC/ISOO guidelines in the management of oral mucositis (Keefe et al. 2007, McGuire et al. 2013, Lalla et al. 2014). Moreover, normal saline and sodium bicarbonate mouth washes may help maintaining oral hygiene, as they are harmless and improve patient comfort (McGuire et al. 2013). However, we and others have shown that the use of antimicrobial mouth rinses for oral mucositis are not recommended, as they do not significantly prevent mucositis or reduce infection risk. On the contrary, they can negatively impact the oral microbial community, which may in turn lead to an unbalanced microbiome (section 3.2)

Not only the microbiota themselves, but also their **secretion products** can enhance or reduce wound healing capacity (Wilson and Gibson 1997, Laheij et al. 2013). **Short chain fatty acids** (SCFA) are able to enhance wound healing of colonic epithelial cells (Wilson and Gibson 1997) and to reduce intestinal permeability and gastrointestinal mucositis in mice (Ferreira et al. 2012). In contrast, De Ryck et al. (2015) showed that accumulation of (a) microbial metabolite(s) is responsible for a reduction in wound healing and **quorum sensing** molecules were thought to be involved in this process. Microbiota use quorum sensing molecules (autoinducers) to regulate gene expression of cell-density dependent processes such as biofilm formation, antibiotic production and virulence. Gram-negative bacteria use acetylated homoserine lactones, whereas Gram-positive bacteria use processed oligopeptides (Miller and Bassler 2001). Moreover, quorum sensing molecules allow communication between bacteria and their host (Hughes and Sperandio 2008). As microbial load and quorum sensing seems important during the wound healing phase of mucositis, quorum sensing inhibitors (Antunes et al. 2010b, Defoirdt et al. 2013) could be an interesting lead to explore in the context of mucositis in the future. By targeting virulence instead of viability of microbial species, problems such as increasing antibiotic resistance could be solved (ten Cate and Zaura 2012).

We suggested in this thesis that the **host** is a crucial intermediate in chemotherapy-induced microbial shifts (section 3.1 and Chapter 3). Before the start of membrane disruptions, activation of the innate immune system and further production of pro-inflammatory cytokines are the first steps in the development of mucositis and are initiated by the cancer treatment (Sonis 2007, Vasconcelos et al. 2016). Following disruption of the epithelial barrier not only DAMPs, but also MAMPs may increase inflammation by NF-κB activation in a TLR-dependent manner (Vasconcelos et al. 2016). The changed host environment characterized by a high

inflammatory status may shift the microbiome both before and following barrier disruption. This disturbed microbiome might impact the recovery from mucositis, as the **microbial community composition** is another factor which may impact wound healing capacity for patients (Chapter 4). Our data confirmed previous research stating that wound healing is not only density dependent, but also species dependent (De Ryck et al. 2015). An unbalanced microbiome might therefore lead to a slow recovery from mucositis, but more research on which species will improve or delay wound healing is needed. The importance of the host and its immune system in creating these microbial shifts during mucositis is similar to other oral diseases. The shift from gingivitis to periodontitis with co-occurring microbial shifts only takes place when a high microbial load is combined with an increased inflammatory status, which creates a niche for anaerobes (Meyle and Chapple 2015, Kilian et al. 2016). Treatment approaches should therefore also focus on the re-establishment of a balanced microbiome.

Different **plaque hypotheses** were described in the last centuries and every oral disease can be linked to multiple of these hypotheses (Rosier et al. 2014). Based the findings described above, mucositis can be linked to three of these hypotheses. First, the 'updated non-specific plaque hypothesis', in which the density of the microbiota is crucial in combination with the fact that resident oral microbiota can become virulent (Theilade 1986). Next, Marsh (1994) linked the overgrowth of pathogenic bacteria to an imbalance of the oral ecosystem caused by an ecological stress in the 'ecological plaque hypothesis'. Additionally he stated the inverse, that bacteria can influence the environment (Marsh 1994). The most recent 'Keystone-pathogen hypothesis', added the importance of the host, its immune system and low-abundant microbial pathogens (Hajishengallis et al. 2012, Rosier et al. 2014).

In conclusion, although the exact role of microbiota is still not clear, we hypothesize that chemotherapy damages the host by disrupting the mucosal barrier, which makes it in turn more susceptible to infections (Vasconcelos et al. 2016). Local infections can aggravate the mucositis process and as most of the cancer patients are immunocompromised, systemic infections could have major consequences. Therefore, we propose that improvement of the **mucosal barrier** has to be the **first** major goal of mucositis treatment, as it reduces the risk of infection. In the past, different strategies targeting specific microbial groups with antimicrobial agents were investigated for **oral mucositis** without any success. As also commensal bacteria (f.e. streptococci) can induce infections and multiple infectious pathogens are known, targeting the pathogenic microbiota does not seem a proper approach. A better option to speed up the wound healing process, is to keep the bacterial load low enough in oral mucositis by mechanical removal of bacteria. Although it is an old method, it is still today the most efficient way of preventing diseases in the oral cavity as it reduces the bacterial load, without disturbing the community composition (Rosier et al. 2014). For **gastrointestinal mucositis**, keeping the microbial load low, without disturbing the entire community, is practically less feasible.

Therefore, the use of anti-inflammatory or immune-suppressive agents, shown to establish mucosal healing in inflammatory bowel disease (De Cruz et al. 2013), could be a possible treatment option in restoring mucosal barrier in gastrointestinal mucositis. Probiotics should be used with extra care due to neutropenia periods in cancer patients, but the use of prebiotics to restore the barrier function might be of interest and will be further discussed in the next section.

Once the mucosal barrier is restored, the **second step** should be to **restore the microbiome** as the cancer treatment indirectly also induces microbial shifts. In our *in vivo* study, we reported that the oral microbiome recovers spontaneously to its initial composition after finishing all chemotherapeutic treatments in the oral cavity of pediatric patients treated for hematological malignancies (Chapter 5). However, a sustained lower microbial diversity was observed in the follow-up samples, indicating that extra measures could help for a full and fast recovery. Possible new treatment approaches such as pre- and probiotics could be investigated in this perspective and will be further discussed in next section. Another approach might be a **preventive treatment** with pre- and probiotics targeting a balanced and diverse microbiome before the start of chemotherapy, in order to prevent disturbances of the microbiome during cancer treatment. Both for oral diseases (Bizzarro et al. 2016) and gastrointestinal diseases (Michail et al. 2012) higher microbial diversity has been linked with better clinical treatment outcomes. Further, a **personalized approach** is possibly required due to the large interindividual differences in the microbiome, the chemotherapeutic regimen, the catabolism of chemotherapeutic agents (for example DPD deficiency), the activity of the host immune system, etc.

6.2 Future treatment options

Probiotics are defined as ‘a live organism that, when ingested in adequate amounts, exerts a health benefit to the host’ and are promising treatment options for many gut diseases (Whelan and Quigley 2013). They exert many beneficial functions on pathways which are important in mucositis and thereby makes them an interesting treatment approach to study. Their mechanisms of action depend on the strain, but include 1) restoration the mucosal barrier, by increasing tight junctions, enhancing epithelial function or stimulating mucin secretion, 2) modulation of the inflammatory immune response by interaction with signaling pathways such as NF-κB and MAPK, thereby influencing cytokine profiles, and 3) displacement of pathogenic bacteria (Lebeer et al. 2010, Thomas and Versalovic 2010, van Vliet et al. 2010, Whelan and Quigley 2013, Vasconcelos et al. 2016).

Most of the research concerning the use of probiotics for mucositis has focused on **gastrointestinal mucositis**. Promising results with *Lactobacillus* and *Streptococcus* strains have been shown in pre-clinical models of chemotherapy-induced mucositis. However, varying effects are observed depending on the model, the strain, the dosing and the treatment plan

(Touchefeu et al. 2014). Clinical trials with probiotics in chemotherapy-induced mucositis are limited so far, although some studies showed that probiotics exerted positive effects on the gut and its microbiome (Osterlund et al. 2007, Wada et al. 2010). More studies have been performed for radiotherapy-induced mucositis, but inconsistent results point to the importance of finding the correct strain and dose for each clinical setting (Touchefeu et al. 2014, Ciorba et al. 2015). Although the effectiveness of probiotics is shown in other gastrointestinal disorders, like inflammatory bowel disease (IBD), also there, recommendations are needed for specific strains and specific symptoms (Whelan and Quigley 2013).

For **oral mucositis**, clinical trials concerning the use of probiotics are even more limited. However, some promising results are shown with *Lactobacillus brevis* CD2 lozenges (Sharma et al. 2012) and AG013, an oral rinse containing recombinant *Lactococcus lactis* secreting mucosal protectant human trefoil factor 1 (hTFF1) (Limaye et al. 2013) as both reduced the severity of oral mucositis. In other oral diseases, the interest in probiotics has also grown the last decades and studies are supportive for the use of probiotics for periodontitis and gingivitis. However, more clinical studies are necessary (Haukioja 2010, Gruner et al. 2016).

Some **safety concerns** must be noted towards the use of probiotics for both gastrointestinal and oral mucositis, as most of the patients are immunocompromised due to the disease and/or the treatment (Touchefeu et al. 2014). In combination with the increased permeability and lesions during mucositis, the risk of infection is very high. As also commensal microbiota, such as viridans streptococci can cause systemic infections, one should be very cautious with the use of probiotics in immunocompromised patients. Although the use of probiotics in immunocompromised patients is shown to be safe (Van den Nieuwboer et al. 2014), translocation and infections by probiotics in immunocompromised patients have been reported (Liong 2008). Therefore, the use of probiotics is probably best advised as a preventive measure for mucositis, before the start of chemotherapy and co-occurring neutropenia, or as a post-treatment option to facilitate recovery to a balanced microbiome (Vasconcelos et al. 2016).

Alternative options without generating a risk for invasive infections could be considered, such as the use of bacterial parts or prebiotics. The use of **bacterial parts** (such as immunostimulatory DNA sequences) has been shown to be sufficient to reduce inflammation for colitis (Rachmilewitz et al. 2002, Borchers et al. 2009). However, no studies in the context of mucositis were conducted so far. The concept of **prebiotics** was first described in 1995 (Gibson and Roberfroid 1995) and defined as ‘a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health’ (Roberfroid 2007). Although largely investigated in gastrointestinal diseases, only a few studies were performed on their effectiveness in mucositis (Smith et al. 2008, Baffoni et al. 2012). However, prebiotics may

impact the microbiota, the mucus layer, the immune system and inflammatory status, without adding an extra bacterial load, and makes them therefore promising candidates for treatment of gastrointestinal mucositis (Wang et al. 2016). Also in the oral cavity, nutritional stimulation of beneficial microbiota by prebiotics could improve recovery of a balanced microbiome (Slomka et al. 2017). As the area of prebiotics is largely underexplored and preliminary research is promising, this might be an interesting way to go in the future for mucositis treatment.

7. Conclusions

The oral and gastrointestinal microbiota are gaining more and more importance in the development of mucositis. As treatment options are elusive and incidences of chemotherapy-induced mucositis are still high, a better knowledge of the impact of chemotherapy on the host microbiome is eligible. By using *in vitro* technologies and a clinical study in pediatric patients suffering from mucositis, we have shown that:

- 5-FU sensitivity varies among oral microorganisms at physiologically relevant concentrations and this is not mediated by dihydropyrimidine dehydrogenase;
- both 5-FU and irinotecan (SN-38) have limited impact on the colon microbial activity and composition;
- oral microbiota reduce wound healing of epithelial monolayers, irrespective of the presence of 5-FU;
- wound healing is inversely correlated with bacterial load for healthy individuals, whereas this is not the case for patients suffering from mucositis probably due the higher abundance of pathogenic genera combined with a less diverse microbial community;
- major patient-specific shifts in the oral microbial community of pediatric patients treated for hematological malignancies occur during and after chemotherapeutic treatment, but partial recovery is observed at the follow-up;
- chemotherapy and mucositis are only correlated with microbial shifts to a minor extent, whereas the use of antibiotics has a major impact and also neutropenia, inflammation and the use of antibacterial mouth rinse with chlorhexidine are correlated with microbial changes.

These findings do not directly translate to a new treatment approach, but they highly encourage good oral hygiene during cancer treatment. Furthermore, they indicate the importance of the host and its immune system in the establishment of chemotherapy-induced microbial shifts.

Summary - Samenvatting

Summary

Although chemotherapy exists already for more than 75 years, it is still associated with multiple side effects. A predominant side effect is mucositis, an inflammation and ulceration of the mucosa that can occur along the entire alimentary tract. Both oral and gastrointestinal mucositis majorly impact the quality of life of cancer patients. Oral mucositis can cause problems with eating, speaking and drinking and is characterized by oral pain, erythema, edema and ulcerations causing a higher infection risk. Symptoms of gastrointestinal mucositis include abdominal pain, diarrhea, constipation, bleeding and infections. Both types of mucositis may lead to a reduction or delay of cancer treatment and unfortunately good treatment options are elusive. A mounting body of evidence suggests a key role for the microbiota in mucositis development. However, the underlying mechanisms remain unclear. Microbial shifts have been observed following chemotherapy in both clinical and animal studies. However, it is not clear whether chemotherapy directly induces microbial shifts or if chemotherapy causes a disturbed host environment inducing microbial changes. In this thesis, we focused on two commonly used chemotherapeutic agents with high incidence of mucositis: 5-fluorouracil (5-FU) and irinotecan (SN-38).

In the first part of this research, the direct effect of chemotherapy on microbiota was investigated. In **Chapter 2**, the effect of physiologically relevant concentrations of 5-FU on the viability and growth of oral bacterial monocultures was investigated. 5-FU sensitivity varied among the tested oral species. *Klebsiella oxytoca*, *Streptococcus salivarius*, *Streptococcus mitis*, *Streptococcus oralis*, *Pseudomonas aeruginosa* and *Lactobacillus salivarius* appeared to be highly resistant to all tested concentrations (0.1-50 µM). In contrast, *Lactobacillus oris*, *Lactobacillus plantarum*, *Streptococcus pyogenes*, *Fusobacterium nucleatum* and *Neisseria mucosa* showed a significant reduction in growth and viability starting from very low concentrations (0.2–3.1 µM). We also provided evidence that dihydropyrimidine dehydrogenase, an enzyme involved in 5-FU resistance in humans, is not involved in the 5-FU resistance of the selected species.

To assess the direct impact of chemotherapeutic agents on a complex microbial ecosystem, we used the M-SHIME®, an *in vitro* mucosal simulator of the human intestinal microbial ecosystem (**Chapter 3**). The direct impact of 5-FU and SN-38 on the luminal and mucosal gut microbiota from several human donors was investigated. At a dose of 10 µM, 5-FU impacted the functionality and composition of the colon microbiota to a minor extent. Similarly, a daily dose of 10 µM SN-38 did not cause significant changes in the functionality or microbiome composition. As our mucosal model does not include a host compartment, we

therefore assume that the changed microbiome observed *in vivo* is primarily induced by an altered host environment upon chemotherapeutic treatment.

In the second part of this research, the interactions between the host, the microbiome and chemotherapeutic treatments were investigated in more detail. In **Chapter 4**, the effect of 5-FU was assessed in an *in vitro* co-culture model that consists of an epithelial cell layer and a biofilm derived from oral microbiota from different oral regions (saliva, buccal and tongue swabs) and donors (healthy individuals and patients suffering from mucositis). Oral microbiota reduced wound healing capacity of epithelial cells with higher bacterial cell counts linked to lower wound healing capacity in healthy individuals. However, for patients suffering from mucositis wound healing was more related to microbial composition, rather than microbial load. Indeed, these oral samples were characterized by a disturbed microbial community and higher abundances of pathogenic genera. However, no major impact of 5-FU on wound healing capacity or the composition of the microbiome was seen. These results emphasize the importance of controlling bacterial load by oral hygiene for proper oral wound healing in healthy individuals. However, extra measures besides oral hygiene might be necessary to assure a good wound healing during mucositis.

To assess the impact of chemotherapy *in vivo*, a longitudinal study of the oral microbiota from five pediatric patients, treated with chemotherapy for hematological malignancies and suffering from oral mucositis, was performed in **Chapter 5**. Microbial community composition analysis showed that large microbial dynamics were present throughout therapy in all patients, however shifts were patient-specific. Mucositis lesions were highly dominated by *Streptococcus*, but also by more pathogenic genera as *Aggregatibacter, Enterococcus* and *Fusobacterium*. Surprisingly, chemotherapy and mucositis had only a minor effect on microbial community composition, whereas one of the major confounding factors of our study was the use of systemic antibiotics as it majorly affected both microbial composition and diversity. Other confounding factors were sample type and sampling period, but also the use of antibacterial mouth rinse with chlorhexidine, neutropenia and inflammation. While the overall community composition seemed to return to its initial composition at least 1 month after therapy, a sustained impact towards a lower diversity was noted. This indicates the importance of long-term follow-up of oral health and good oral hygiene for these patients.

In conclusion, this PhD research demonstrated that the direct effect of chemotherapy on the oral and gut microbiome is limited, but that the chemotherapy-disturbed host environment may largely impact the host microbiota. A low bacterial load may improve wound healing capacity and reduce risk of infection. Mechanical removal of oral microbiota is preferred, as antimicrobial rinses may cause microbial shifts leading to dysbiosis. However, long-term follow-up and extra measures are needed to assess a fast and full recovery of both host mucosa and microbiota in chemotherapy-induced mucositis.

Samenvatting

Chemotherapie bestaat al meer dan 75 jaar, maar gaat nog steeds gepaard met veel neveneffecten. Een van de belangrijkste bijwerkingen is mucositis, die wordt gekenmerkt door een ontsteking en aften ter hoogte van de slijmvliezen van het spijsverteringsstelsel. Zowel orale als gastro-intestinale mucositis kunnen de levenskwaliteit van de patiënt sterk beïnvloeden. Oral mucositis kan problemen veroorzaken met spraak en voedselopname en wordt typisch gekenmerkt door pijn, roodheid, zwelling en ontstekingen in de mond, wat kan leiden tot een verhoogd risico op lokale en systemische infecties. De belangrijkste symptomen van gastro-intestinale mucositis daarentegen omvatten buikpijn, diarree, constipatie, bloedingen en infecties. Beide types van mucositis kunnen leiden tot een dosisreductie of zelfs uitstel van de kankertherapie. Tot op heden bestaan er nog steeds geen afdoende behandelingen voor mucositis.

Recent onderzoek duidt een belangrijke rol aan voor de bacteriën in de darm en de mond bij de ontwikkeling van mucositis, al blijven de onderliggende mechanismen onduidelijk. Zowel klinische studies als dierenproeven hebben aangetoond dat zich veranderingen in de microbiële gemeenschap voordoen tijdens/na een behandeling met chemotherapie. Het is echter niet duidelijk of de chemotherapie zelf de microbiële veranderingen teweeg brengt of de chemotherapie de gastheeromgeving verstoort waardoor veranderingen in de microbiële samenstelling ontstaan. In deze thesis hebben we gefocust op twee courante chemotherapeutica waarvan de behandeling vaak gepaard gaat met het optreden van mucositis, namelijk 5-fluorouracil (5-FU) en irinotecan (SN-38).

In het eerste deel van dit onderzoek werd het direct effect van chemotherapie op bacteriën onderzocht. Het effect van fysiologisch relevante concentraties 5-FU op de viabiliteit en groei van orale bacteriële monoculturen werd onderzocht in **Hoofdstuk 2**. Er werd aangetoond dat de gevoeligheid voor 5-FU varieert tussen de geteste orale species. *Klebsiella oxytoca*, *Streptococcus salivarius*, *Streptococcus mitis*, *Streptococcus oralis*, *Pseudomonas aeruginosa* en *Lactobacillus salivarius* bleken resistent bij alle geteste concentraties (0.1-50 µM). *Lactobacillus oris*, *Lactobacillus plantarum*, *Streptococcus pyogenes*, *Fusobacterium nucleatum* en *Neisseria mucosa* daarentegen vertoonden een significante reductie in groei en viabiliteit al vanaf zeer lage concentraties 5-FU (0.2-3.1 µM). Daarnaast werd aangetoond dat het dihydropyrimidine dehydrogenase enzym dat belangrijk is bij 5-FU resistentie in patiënten, geen rol speelt bij de resistentie bij de geteste bacteriële species.

In **Hoofdstuk 3** maakten we gebruik van de M-SHIME®, een *in vitro* model voor de humane colon bacteriën, om de directe impact van chemotherapeutica na te gaan op een complex microbieel ecosysteem. Het effect van 5-FU en SN-38 op luminale en mucosale

darmbacteriën werd onderzocht voor verschillende donoren. Daaruit bleek dat een dosis van 10 µM 5-FU slechts een beperkte invloed had op de functionaliteit en de samenstelling van de colon bacteriën. Ook een dagelijkste dosering van 10 µM SN-38 had geen significante impact op de microbiële functionaliteit of samenstelling. Aangezien het *in vitro* model geen gastheer compartiment omvat, veronderstellen we dat *in vivo*, het de verstoring van de gastheeromgeving is die zal leiden tot veranderingen in het microbioom.

In een tweede deel van dit onderzoek, werden de interacties tussen de gastheer, de bacteriën en chemotherapie meer in detail bestudeerd. In **Hoofdstuk 4** werd het effect van 5-FU onderzocht in een *in vitro* co-cultuur model bestaande uit een epitheliale cellaag en een biofilm afkomstig van orale bacteriën van verschillende orale regio's (speeksel, wang- en tongswabs) en verschillende donoren (gezonde individuen en patiënten met orale mucositis). Onze resultaten toonden aan dat orale bacteriën de wondhelingscapaciteit van de epitheliale cellen reduceren en dat hogere bacteriële celaantallen zorgen voor een lagere wondhelingscapaciteit in gezonde individuen. Voor patiënten met orale mucositis daarentegen zijn niet alleen de celaantallen belangrijk, maar de ook de microbiële samenstelling, aangezien pathogene genera in hogere relatieve hoeveelheden aanwezig waren. Verrassend, 5-FU vertoonde geen significant effect op wondheling noch op de microbiële samenstelling. Deze resultaten benadrukken het belang van orale hygiëne voor een goede wondheling bij gezonde individuen, maar wijzen er ook op dat extra maatregelen nodig kunnen zijn bij patiënten met mucositis.

Verder werd een opvolgingsstudie uitgevoerd, waarbij gekeken werd naar de samenstelling van de orale bacteriën afkomstig van vijf pediatrische kankerpatiënten om de impact van chemotherapie *in vivo* na te gaan (**Hoofdstuk 5)**. De patiënten werden behandeld met chemotherapie voor een hematologische aandoeding en ontwikkelden allemaal orale mucositis. Voor alle patiënten werden verschuivingen waargenomen in de orale microbiële samenstelling tijdens de therapie, al bleken deze patiënt-specifiek. Mucositis letsels werden vooral gedomineerd door streptococci, maar ook door meer pathogene genera zoals *Aggregatibacter*, *Enterococcus* en *Fusobacterium*. Chemotherapie en mucositis hadden verrassend genoeg slechts een kleine impact op de samenstelling van de orale bacteriën. Daarentegen was het gebruik van systemische antibiotica een van de belangrijkste factoren in onze studie, aangezien het een grote impact had op zowel de samenstelling als de diversiteit van de orale bacteriën. Daarnaast werden ook het staaltype, de periode van staalname, het gebruik van antibacteriële mondspoelingen met chloorhexidine, neutropenie en inflammatie gelinkt met verschuivingen in de microbiële samenstelling. Minstens 1 maand na de therapie, herstelde de microbiële samenstelling zich terug naar de initiële samenstelling, al was de diversiteit beduidend lager. Dit duidt opnieuw op het belang van een goede opvolging van de patiënt en van een goede orale hygiëne.

In dit doctoraatsonderzoek werd aangetoond dat het direct effect van chemotherapie op de orale en gastro-intestinale bacteriën beperkt is. Daarentegen zal chemotherapie vooral het volledige gastheerweefsel verstoren wat vervolgens aanleiding kan geven tot grote verschuivingen in het microbioom. Het beperken van de microbiële belasting kan wondheling stimuleren en het risico op infecties verlagen. Bij orale mucositis is hierbij een mechanische verwijdering van orale bacteriën aangeraden, aangezien antibacteriële mondspoelingen verschuivingen in het microbioom kunnen teweeg brengen en aanleiding kunnen geven tot dysbiose. Echter, een langdurige opvolging met eventuele extra maatregelen zijn aan te raden om een volledig herstel van zowel de slijmvliezen als de bacteriën te bewerkstelligen na mucositis en om het risico op infecties te beperken.

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Scientific Curriculum Vitae

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Education

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Publications

Articles on ISI Web of Science (published, A1)

1. **Vanlancker, E.**, Vanhoecke, B., Smet, R., Props, R. and Van de Wiele, T. (2016). 5-Fluorouracil sensitivity varies among oral microorganisms. *Journal of Medical Microbiology,* **65**, 775-783.
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1. **Vanlancker, E.**, Vanhoecke, B., Stringer, A. and Van de Wiele, T. 5-Fluorouracil and irinotecan (SN-38) have limited impact on colon microbial functionality and composition in vitro. *Submitted at PeerJ.*
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3. **Vanlancker, E.**, De Vrieze, J., Vanhoecke, B., Desmaele, L., De Moerloose, B. and Van de Wiele, T. Longitudinal analysis of oral microbiota during chemotherapy-induced mucositis in pediatric patients. *In preparation.*

Participation at international conferences

Oral presentations

1. **Vanlancker**, **E.**, Van de Wiele, T. and Vanhoecke, B. Host-microbe interactions in chemotherapy-induced oral mucositis. Presented at: 18th Gut Day Symposium (Venlo, The Netherlands, November 27th, 2016).
2. **Vanlancker, E.**, Stringer, A., Van de Wiele, T. and Vanhoecke, B. A new *in vitro* model to study host-microbe interactions in chemotherapy-induced mucositis. Presented at: 21st

National Symposium for Applied Biological Sciences (Antwerp, Belgium, February 5th, 2016).

3. **Vanlancker, E.**, De Ryck, T., Stringer, A., Merca, E., Van de Wiele, T., and Vanhoecke, B. A new *in vitro* model to study host-microbe interactions in chemotherapy-induced mucositis. Presented at: 16th Gut Day Symposium (Amsterdam, The Netherlands, November 27th, 2014).

Poster presentations

1. **Vanlancker, E.**, Van de Wiele, T. and Vanhoecke, B. Host-microbe interactions in chemotherapy-induced oral mucositis. Presented at: IRC Symposium 2016 (Ghent, Belgium, September 14th, 2016).
2. **Vanlancker, E.**, Van de Wiele, T., and Vanhoecke, B. Host-microbe interactions in chemotherapy-induced oral mucositis. Presented at: 9th MASCC/ISOO International symposium on supportive care in cancer (Adelaide, Australia, June 23rd-25th, 2016).
3. **Vanlancker, E.**, Vanhoecke, B., Stringer, A. and Van de Wiele, T. 5-Fluorouracil and irinotecan (SN-38) do not modulate the gut microbiome composition and activity in the absence of a host system. Presented at: 9th MASCC/ISOO International symposium on supportive care in cancer (Adelaide, Australia, June 23rd-25th, 2016).
4. **Vanlancker E.**, De Ryck, T., Stringer, A., Merca, E., Van de Wiele, T. and Vanhoecke, B. A new *in vitro* model to study host-microbe interactions in chemotherapy-induced mucositis. Presented at: 8th MASCC/ISOO International symposium on supportive care in cancer (Copenhagen, Denmark, June 25th-27th,2015).
5. **Vanlancker, E.**, Vanhoecke, B. and Van de Wiele, T. Short-term effects of a single dose of 5-fluorouracil on gut microbiota. Presented at: 16th Gut Day Symposium (Amsterdam, The Netherlands, November 27th, 2014).
6. De Ryck, T., **Vanlancker, E.**, Boterberg, T., Bracke, M., Van de Wiele, T. and Vanhoecke, B. Host-microbioma crosstalk in cancer therapy. Presented at: 7th MASCC/ISOO International symposium on supportive car in cancer (Miami, USA, June 26th-28th, 2014).
7. **Vanlancker, E.**, Stringer, A., De Ryck, T., Merca, E., Van de Wiele, T. and Vanhoecke, B. A new *in vitro* model to study host-microbe interactions in chemotherapy-induced mucositis. Presented at: Knowledge for growth 2014 (Ghent, Belgium, May 8th,2014).
8. De Ryck, T., **Vanlancker, E.**, Boterberg, T., Bracke, M., Van de Wiele, T. and Vanhoecke, B. Microbe-epithelial crosstalk in cancer therapy. Presented at: Knowledge for growth 2014 (Ghent, Belgium, May 8th, 2014).

9. De Ryck, T., **Vanlancker E.**, Boterberg, T., Bracke, M., Van de Wiele, T. and Vanhoecke, B. Microbe-epithelial crosstalk in cancer therapy. Presented at: Belgian Association for Cancer Research (BACR) Annual Meeting (Zwijnaarde, Belgium, February 2014).
10. De Ryck, T., **Vanlancker, E.**, Boterberg, T., Bracke, M., Van de Wiele, T. and Vanhoecke, B. Microbe-epithelial crosstalk in cancer therapy. Presented at: Oncopoint Meeting (Ghent, Belgium, February 2014).
11. **Vanlancker, E.**, De Ryck, T., Vanhoecke, B. and Van de Wiele, T. Effect of oral microbiota on epithelial wound healing in the context of oral mucositis. Presented at: 15th Gut Day Symposium (Groningen, The Netherlands, November 7th, 2013).
12. Vanhoecke, B., De Ryck, T., **Vanlancker, E.**, Bowen, J., Van de Wiele, T. and Keefe, D. Development of a multidisciplinary platform for the screening of new preventive and therapeutic drugs against mucositis. Presented at: 6th MASCC/ISOO International symposium on supportive care in cancer (Berlin, Germany, June 27th-29th, 2013).

Teaching

1. Responsible for practical exercises for the course 'Host-microbe interactions' (Prof. Tom Van de Wiele) (2013-2016)
2. Tutor of 1 bachelor and 3 master students during their thesis (2014-2017)
3. Teaching STEM project for Atheneum Zottegem (2017)

Awards

1. Best oral presentation, 18th Gut Day Symposium, November 27th, 2016, Venlo, The Netherlands.

Dankwoord - Acknowledgements

Na vier jaar is het eindelijk zover, tijd om de laatste bladzijden van mijn doctoraat te schrijven. Een doctoraat schrijven doe je echter niet alleen en daarom wil ik graag nog enkele mensen bedanken.

Eerst en vooral zou ik de leden van mijn jury willen bedanken voor hun constructieve feedback en boeiende discussies tijdens mijn verdedigingen. Jullie kritische bijdrage vanuit zeer diverse wetenschappelijke achtergronden heeft mijn thesis verder geoptimaliseerd, waardoor het toegankelijk is voor een breed publiek.

Een doctoraat is geen doctoraat zonder promotoren en ik had het geluk twee fantastische promotoren aan mijn zijde te hebben gedurende deze vier jaar. Bedankt allebei om me vier jaar geleden de kans te geven om een doctoraat te starten op CMET. *Tom*, ik wil jou graag bedanken voor de vele steun, input en boeiende discussies. Je stond altijd klaar met een luisterend oor en de nodige tips als er iets fout was gelopen in het labo (zoals mijn cellen die weer eens niet wouden groeien). Je was er steeds om me in de juiste richting verder te leiden en jouw enthousiasme op mij over te dragen wanneer te resultaten soms anders uitdraaiden dan we hadden verwacht. Kortom, ik heb heel veel van jou bijgeleerd, als onderzoeker en als mens, merci! *Barbara*, ook al was je in het begin van mijn doctoraat aan de andere kant van de wereld, toch kon ik steeds rekenen op jouw input en enthousiaste feedback via onze vele skype gesprekken, bedankt hiervoor. Ik kijk ook met veel plezier terug op jouw verblijf hier op CMET in 2015 toen we samen in het labo hebben gestaan. Toen waren de rollen soms omgekeerd en leerde ik jou alle kneepjes van de SHIME kennen, maar ik heb toch vooral veel van jou geleerd tijdens deze vier jaar. Dankzij jou had ik ook de gelegenheid om naar Adelaide te gaan, bedankt voor deze fantastische ervaring!

I would like to thank the people I worked with in Adelaide. *Andrea*, *Bronwen* and all other colleagues, thank you for giving me the opportunity to be part of your lab for a couple of weeks, for all the help in the lab, the interesting discussions at the MASCC/ISOO conference, for showing me around Adelaide and surroundings and to let me feel at home in Australia. It was an amazing experience!

Daarnaast zijn er ook heel wat andere mensen die ik wil bedanken voor de fijne samenwerking tijdens mijn doctoraat. *Prof. De Moerloose*, bedankt voor de hulp bij de patiëntenstudie en de interessante discussies omtrent mucositis. *Maaike*, *Rein*, *Annelore* & *Véronique*, heel erg bedankt voor de afname van alle patiëntenstalen, zonder jullie had deze studie niet mogelijk geweest. Ik mag ook zeker de patiëntjes en hun ouders niet vergeten: bedankt om in vaak moeilijke tijden deel te nemen aan mijn studie. Hartelijk dank ook aan *Prof. De Vos* en *Tom* voor het gebruik van de microscoop en de vele hulp bij de uitvoering en analyse van mijn wondhelingsexperimenten. *Charlotte*, bedankt voor het gebruik van jullie

cellabo in het begin van mijn doctoraat en de vele boeiende discussies later in clusters en meetings.

Dit onderzoek is mede tot stand gekomen met de hulp van enkele studenten tijdens hun thesis. *Rozel*, *Janie*, *Laura* en *Annelore*, jullie waren één voor één voortreffelijke studenten en ik kijk met veel plezier terug op onze samenwerking.

Zonder het ATP, draait het labo natuurlijk niet, daarom zou ik ook graag hen bedanken voor hun hulp. *Christine*, *Regine & Sarah*, bedankt voor de vele praktische regelingen en administratie. *Greet*, *Siska*, *Mike*, *Renée*, *Tom* en *Annick*, bedankt voor alle hulp in het labo en de gezellige lunchpauzes. *Tim*, bedankt voor de hulp en tips bij het (moleculair) labowerk, de mooie figuren in mijn presentatie en de plezante drinks in de Koepuur. *Jana*, bedankt voor de vele hulp in het labo, maar vooral voor de vriendschap. We hebben samen veel herinneringen opgebouwd in de afgelopen vier jaar, met als hoogtepunten Italië, Thailand en vele fijne momenten samen in de Maaltebruggestraat. Ik ben zeker dat we hier nog veel mooie herinneringen aan gaan toevoegen in de toekomst.

I big thank you to all HAM-cluster members, for the nice clusters, the fruitful discussions, the interesting input and the nice atmosphere. Some special thanks to: - *Tine*, bedankt om me tijdens mijn masterthesis zo goed te begeleiden dat ik zin had om nog vier jaar verder te gaan met dit onderzoek - *Annelies*, bedankt om me zo goed op te vangen in het begin van mijn doctoraat en voor de fijne vriendschap die hieruit is ontstaan - *Kim* & *Floor*, het was tof om samen met jullie een doctoraat te starten eind 2013. Bedankt voor de fijne momenten in en ook buiten het labo - *Rosemarie*, het was zeer fijn samen met jou het cellabo op CMET op te starten en je was een fantastische buurvrouw op het bureau - *Marta* & *Charlotte*, it was great to work with you in the celllab - *Ioanna*, thanks for the nice collaboration concerning the oral microbiome and the use of mouth washes.

I also would like to thank my office-mates for the nice time I had with them. *Jan*, *Jo*, *Marlies*, *Rosemarie*, *Ramon*, *Pieter*, *Jose*, *Charlène*, *Annelies*, *Samuel*, *Bernabé*, *Huajun* and *Eva*, I had a great time in our office and really enjoyed the nice atmosphere, the lunch breaks and great office-activities.

Many thanks also to the CMET running team (*Jan*, *Tim*, *Jo*, *Marlies*, *Charlotte*, *Sylvia*, *Amanda*, *Melanie*, *Ramon*, *Pieter*, *Jose, Erika* …) for the nice afterwork-runs and (Ghent) city runs. Thanks to you I always looked forward to go for a run. *Sylvia* & *Marlies*, naast samen lopen maakten we ook tijd voor gezellige avonden onder vriendinnen, bedankt!

To all other CMET (ex-)colleagues: thank you for your support and help during my PhD and for the nice coffee breaks, lunch breaks and (Koepuur) drinks. It has been four amazing years with you all!

Ook naast het labo waren er heel wat mensen die steeds achter mij stonden en mij steunden. Ik wil hiervoor graag nog enkele mensen bedanken.

Emmily, *Tine*, *Caro*, *Jolijn*, *Joni* en *Nabila*, ook wel 'Captain Tomislav crazy crew', als enige ingenieur, kon ik niet anders dan ook de titel 'doctor' te behalen. Al heeft het bij mij wel negen in plaats van zeven jaar geduurd ;-). Bedankt voor de antwoorden op mijn vele medische vragen, de gezellige avonden samen koken, de plezante uitstapjes en de toffe weekends en reizen samen. *Emmily* en *Caro*, bedankt ook om me te blijven aanmoedigen om samen te sporten tijdens de laatste maanden van mijn doctoraat.

Daarnaast kan ik ook steeds rekenen op een hele groep Chiro vriendinnen. Ook als oudleiding blijven we een hechte groep vormen, ons zalig amuseren samen en onvergetelijke momenten beleven. Bedankt allemaal! *Maggelien*, *Tine*, *Tine*, *Gene, Sofie, Tess, Melissa* & *Deli*, 20 jaar geleden gingen we voor het eerst samen op kamp en we hebben sindsdien al vele mooie herinneringen verzameld. Onze skireis samen gaf me de nodige energie om de laatste maanden scrhrijven tegemoet te gaan en met ons weekendje Berlijn hebben we alweer iets om naar uit te kijken samen, merci!

Naast steun van collega's en vrienden, kon ik ook steeds rekenen op mijn *familie*. Vol interesse vroegen jullie steeds hoe het was met mijn doctoraat en wat ik al had 'ontdekt'. Ik hoop dat ik jullie hierop vandaag een antwoord heb kunnen geven. Trots en fier, zo kan ik mijn *oma's en opa's* het best omschrijven als ze het hadden over mijn doctoraat. Ikzelf ben vooral trots om hun kleindochter te zijn en blij dat ze zoveel interesse in mijn onderzoek tonen. Opa, het is jammer dat je er vandaag niet meer bij bent om alles met jouw camera in de hand vast te leggen voor het nageslacht, maar ik ben zeker dat ook jij nu fier bent.

En dan kom ik toe aan de belangrijkste mensen in mijn leven, die al meer dan 27 jaar achter mij staan. *Mama* en *papa*, heel erg bedankt voor alle steun, om mij alle kansen te geven en mij een warme thuis te geven. Merci beaucoup! *Tine*, zusje, jij verstaat me als geen ander en staat steeds klaar als ik jou nodig heb. Bedankt voor alles, al mijn hele leven lang.

Elien, liefste schat, bedankt om de laatste twee jaar aan mijn zijde te staan en in mij te geloven. Je bent er steeds voor mij en dat apprecieer ik enorm. Bedankt ook voor de mooie cover en om me achterna te komen naar het verre Australië. Het was een fantastische ervaring met heel veel mooie herinneringen samen.

Eline, 13 oktober 2017